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Food and Drug Administration

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SKIN TEST ANTIGENS

Proposed Implementation of Efficacy Review

[4110–03]

DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE

Food and Drug Administration

[21 CFR Parts 601, 610, and 650]

[Docket No. 76N–0480]

SKIN TEST ANTIGENS

Proposed Implementation of Efficacy Review

AGENCY: Food and Drug Administration.

ACTION: Proposal.

SUMMARY: The Food and Drug Administration (FDA) is proposing to amend the biologics regulations in response to the report of the Advisory Review Panel on the safety, effectiveness, and labeling of skin test antigens. Additionally, on the basis of the Panel's findings and recommendations, the Commissioner of Food and Drugs is announcing his conclusions on those products in Category II (unsafe, ineffective, or misbranded) and Category IIIB (off the market pending completion of studies permitting a determination of effectiveness). The licenses for products in Category II have already been revoked at the request of the license holder. On or before October 31, 1977, the Commissioner will publish notices of opportunity for hearing to revoke the licenses for products in Category IIIB. The Commissioner is also announcing his conclusions as to those products in Category I (safe, effective, and not misbranded) and Category IIIA (on the market during further studies in support of effectiveness) and, by this proposal, is inviting comments and the submission of views and additional data on the status of these products.

DATES: Comments by November 29, 1977.

ADDRESS: Written comments to the Hearing Clerk (HFC–20), Food and Drug Administration, Room 4–65, 5600 Fishers Lane, Rockville, Md. 20857.

FOR FURTHER INFORMATION CONTACT:

Al Rothschild, Bureau of Biologics (HFB–620), Food and Drug Administration, Department of Health, Education, and Welfare, 8800 Rockville Pike, Bethesda, Md. 20014 (301–443–1920).

SUPPLEMENTARY INFORMATION: In the FEDERAL REGISTER of February 13, 1973 (38 FR 4319), the Commissioner issued § 601.25 (21 CFR 601.25) concerning procedures for review of safety, effectiveness, and labeling of biological products licensed prior to July 1, 1972. The biological products reviewed were assigned to one of the following categories: (a) Bacterial vaccines and bacterial antigens bearing labeling stating "No U.S. Standards of Potency," (b) bacterial vaccines and toxoids with standards of potency, (c) viral vaccines and rickettsial vaccines, (d) allergenic extracts, (e) skin test antigens, (f) blood and blood derivatives.

Pursuant to § 601.25, the Commissioner assigned responsibility for the initial review of each of the biological product categories to a separate independent advisory panel consisting of qualified experts to insure objectivity of the review and public confidence in the use of these products. The Commissioner charged each panel to (1) evaluate the safety and effectiveness of the biological product, (2) review the labeling of the biological product, and (3) advise him on which biological products under review are safe, effective, and not misbranded, in the form of an advisory review panel report to the Commissioner. The advisory review panel report is to include a statement classifying products into 1 of 3 categories.

Category I designates those biological products determined by the panel to be safe, effective and not misbranded. The panel statement may include any condition relating to active components, labeling, tests required prior to release of batches, product standards, or other conditions necessary or appropriate for their safety and effectiveness.

Category II designates those biological products determined by the panel to be unsafe or ineffective or to be misbranded.

Category III designates those biological products determined by the panel not to fall within either Category I or II on the basis of the panel's conclusion that the available data are insufficient to classify such biological products, and for which further testing is therefore required. Those biological products in Category III for which continued licensing, manufacturing, and marketing are recommended are designated as Category IIIA. Those biological products in Category III for which revocation of the license is recommended (and thus denying continued manufacturing and marketing) are designated as Category IIIB. The recommendation for either Category IIIA or IIIB is based on an assessment of the present evidence of safety and effectiveness of the product and the potential benefits and risks likely to result from the continued use of the product for a limited period of time while the questions raised concerning the product are being resolved by further study.

Subsequently, in the FEDERAL REGISTER of February 26, 1974 (39 FR 7445), the Commissioner requested that interested persons nominate qualified experts to serve on the Panel on Review of Skin Test Antigens. In addition, the Commissioner solicited submission of data pertinent to these products to facilitate review of safety, effectiveness, and labeling of these products and to provide all interested persons an opportunity to present, for consideration by the Panel, the best information available to support the stated claims for skin test antigens.

Data and information submitted pursuant to the February 26, 1974, notice and falling within the provisions of 18 U.S.C. 552(b) or 21 U.S.C. 331(j) have been handled as confidential. However, with the publication of this proposed implementation and the full report of the panel, such data and information will, pursuant to § 601.25(b)(2), be made publicly available October 31, 1977, and may be viewed at the office of the Hearing Clerk, except to the extent that the person submitting the data demonstrates that such data still fall within one or more of the confidentiality provisions. Accordingly, any such comments concerning confidentiality should be submitted by October 31, 1977, to Office of Efficacy Review (HFB–5), Bureau of Biologics, 8800 Rockville Pike, Bethesda, Md. 20014.

The Commissioner appointed the following Panel to review the data and information submitted, and to prepare a report on the safety, effectiveness, and labeling of skin test antigen biological products:

Panel Chairman, John A. Sbarbaro, M.D., M.P.H., Director of Public Health and Deputy Director for Medical Affairs, Denver Department of Health and Hospitals; Associate Professor of Medicine and Preventive Medicine, University of Colorado School of Medicine.

Charlotte C. Campbell, D.Sc., Professor of Medical Sciences, Southern Illinois University School of Medicine.

George W. Comstock, M.D., Professor of Epidemiology, The Johns Hopkins School of Hygiene and Public Health.

Fred S. Kantor, M.D., Professor of Medicine, Yale University School of Medicine.

George S. Kobayashi, Ph.D., Associate Professor, Division of Dermatology, Department of Internal Medicine, Washington University School of Medicine.

John E. Salvaggio, M.D., Henderson Professor of Medicine, Tulane Medical School.

Emanuel Wolinsky, M.D., Professor of Medicine, Case Western Reserve University, Director of Microbiology, Cleveland Metropolitan General Hospital.

The Panel was first convened on June 25, 1974, in an organizaitonal meeting. Working meetings were held on September 20 and 21, October 18 and 19, November 22 and 23, 1974, and January 10 and 11, February 28 and March 1, April 18 and 19, June 6 and 7, August 1 and 2, September 26 and 27, and December 5 and 6, 1975.

Three nonvoting liaison representatives served on the Panel. Ms. Cathy Sulzberger, nominated by the Consumer Action for Improved Food and Drugs organization, served as the consumer representative. John G. Adams, Ph.D., nominated by a number of producers with products under review by the Panel, served as the industry representative. Karl Bambach, Ph.D., substituted for Dr. Adams in his absence.

Clay Sisk, U.S. Public Health Service, served as Executive Secretary to the Panel. Morris Schaeffer, M.D., Ph.D., participated in the Panel meetings in his capacity as Director, Office of Efficacy Review, Bureau of Biologics, Food and Drug Administration.

The following individuals were given an opportunity to appear before the Panel to express their views either at their own or the Panel's request:

Walter R. Ashford, Ph.D.
Sotiros D. Chaparas, Ph.D.
Murray S. Cooper, Ph.D.
Phyllis Q. Edwards, M.D., MPH
Laurence S. Farer, M.D., MPH
Kenneth Herrmann, M.D.
Silvio Landi, Ph.D.
Stephen A. Szumski, Ph.D.

No person who so requested was denied an opportunity to appear before the Panel.

The following firms made submissions relating to the indicated products:

SUBMISSIONS BY FIRMS

Firm	*Licensed products*
Bureau of Laboratories, Michigan Department of Public Health, Lansing, Mich. 48914.	Histoplasmin.
Connaught Laboratories, Ltd., Willowdale, Ontario, Canada.	Tuberculin, PPD.
Cutter Laboratories, Inc., Berkeley, Calif. 94710.	Coccidioidin.
Eli Lilly & Co., Greenfield, Ind. 46140.	Mumps Skin Test Antigen, Tuberculin, Old.
Iatric Corp., Tempe, Ariz. 85282.	Coccidioidin.
Institut Merieux, Lyon (2e), France Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y. 10965.	Tuberculin, Old. Histoplasmin, Lymphogranuloma Venereum Antigen, Trichinella Extract, Tuberculin, Old.
Massachusetts Public Health Biologics Laboratories, Boston, Mass. 02130.	Diphtheria Toxin for Schick Test, Tuberculin, Old.
Parke Davis & Co., Detroit, Mich. 48232.	Histoplasmin, Tuberculin, PPD.
Texas State Department of Health, Austin, Tex. 78756.	Diphtheria Toxin for Schick Test, Schick Test Control, Tuberculin, Old.

No data were submitted on the products listed below for which licenses were in effect as of September 27, 1975, although it appears that these products have not been manufactured since mid-1973.

LICENSED PRODUCTS NOT BEING MARKETED

Firm	*Licensed products*
Parke Davis & Co., Detroit, Mich. 48232.	Diphtheria Toxin for Schick Test, Tuberculin, Old.
E. R. Squibb & Sons, Inc., New Brunswick, N.Y. 08903.	Lymphogranuloma Venereum Antigen.

The Panel has thoroughly reviewed the literature and various data submissions and has considered all pertinent data and information submitted through December 5, 1975, in arriving at the following conclusions and recommendations:

BASIS OF EVALUATION

Recognition of the relationship between microorganisms and the human host is a relatively new development in the long history of mankind. Less than 100 years of intensifying investigations has revealed that contact between man and an infecting agent can range in results from imperceptible and transient, but harmless, multiplication within the host to overwhelming infection resulting in death of the host. Although any microorganism capable of multiplying in man is a potential threat, the vast majority of these agents are quickly subdued by man's natural immune mechanisms. Once having come in contact with the infecting microorganism, the host may become "sensitized" to the infecting agent or components of the infecting agent. This "sensitization" is part of the host immune system. Some microorganisms such as *Mycobacterium tuberculosis* pose a serious and lifelong threat to the host, despite such "sensitization."

The ablity to identify whether an individual has experienced or is experiencing invasion by a specific microorganism is often of medical significance. One of the earliest developed methods through which this relationship between the host and parasite can be ascertained is the injection of cellular components of the suspected microorganism into the skin of the presumed host. The concept underlying this test is relatively simple: Components (antigens) capable of eliciting response by "sensitized" immune defenses of the body should be easily identified when placed in a small confined area of the skin where the reaction by the host defensive system can be measured. A definite response (positive reaction) indicates contact and interaction between the individual and the specific microorganism or an antigenically related one.

Initially, this testing process was grossly crude, involving the use of ill-defined antigens thought to be derived from specific microorganisms but often including components of other substances such as those used to grow or prepare the microorganism for skin injection. While experience and scientific advances have permitted the gradual refining of many of these earlier products, the state of the art still encompasses a wide range of products of varying purity and potency.

In evaluating these products for both safety and effectiveness, the Panel first defined the characteristics of an ideal skin test antigen. A perfect skin test should be sensitive enough to identify every individual who has interacted with a particular microorganism, yet specific enough to identify only those individuals. At least three variables contribute to the effectiveness of the skin test process:

(1) *Immunological system.* The variable introduced by the individual human immunological response system is beyond the scope of this Panel. However, general immune deficiencies and poorly understood findings such as specific anergy (nonreactivity to a specific antigen) are medical realities that complicate this testing process. Gradations of these entities undoubtedly exist, adding further to the impact of this test variable. While of significance in a single setting, these factors should have little effect on the overall test product or on the epidemiological conclusions arising from use of the skin test.

(2) *Test material.* Ideally, the antigen used in a skin test process should be identical from production lot to production lot, an accomplishment that can only be achieved if the exact chemical composition of the antigen has been identified and can be reproduced. Additionally, to insure equality of biological impact by production batches, evidence of identical antigenicity should be confirmed in an animal model proven to correlate with the human system. To be accurate, this animal model requires the existence of a reference standard which has been evaluated in human population groups and against which new production lots can be compared. Data must exist confirming the permanent stability of the reference standard. Similarly, an ideal skin test antigen would remain stable over a defined time, neither changing through chemical decomposition nor adsorption to container walls.

Serious reconsideration must be given to any test material (antigen) which can alter the immunological system when deposited into a small confined area of human skin. Material which causes the development of a lifelong immunological response when there has been no interaction between host and microorganism is unsuitable for skin test use. Such material may be valuable as a vaccine, but not as a diagnostic tool. Even a transient effect on an immune system which has not had experience with the source microorganism is a serious drawback to use of the test material as a skin test antigen.

In addition to not inducing an immune response, the ideal skin test antigen should not augment or boost the reaction resulting from a previous interaction between host and microorganism. Unfortunately, this phenomenon is not unusual and probably results more from the stimulation or re-awakening of a "sensitized" immune system, diminished from years of noncontact with the spefic microorganism, rather than from any characteristic of skin test antigen material itself. Ideally, the antigen deposited into the skin should be capable of being completely eliminated by the host.

Finally, the solution (diluent) into which the testing material is dissolved must neither sensitize the immune system nor elicit any other form of host reaction, local or systemic. These attributes should also characterize the preservative used in the product.

(3) *Test method.* An ideal test method requires the simple and reproducible deposit of an accurate amount of test antigen into a selective skin site of the host. The immune response (reaction) elicited by this antigen should be easily measured; round or sufficiently symmetrical and uniform in shape so that a single diameter is a reliable indicator of reaction size. The boundaries of the reaction should be clear-cut for ease of measurement. Furthermore, the range of reaction sizes should be large enough to permit quantitative dose response studies. The response must remain unchanged for a

defined time, preferably over a 24-hour period, thereby allowing for flexibility in the time for completing the measurement (reading) of the test response.

The conceptualization of these characteristics of the ideal effective skin test is outlined as follows:

IDEAL SKIN TEST ANTIGEN

1. *Product.* The purpose of an ideal skin test antigen is to safely measure skin test reactivity which indicates the presence or absence of specific immunologic experience.

2. *Analysis.*—(a) *Effectiveness.*—The ideal skin test antigen should identify all individuals with specific immunologic experience (sensitivity), and identify only individuals with specific immunologic experience (specificity).

CHARACTERISTICS OF EFFECTIVENESS

(i) Test material:
(1) Defined chemical composition.
(2) Reproducibility from lot to lot.
(3) Standardizable in laboratory system. Laboratory system correlates to system in humans.
(4) Stable.
(5) Nonsensitizing (at dosage recommended):
(*a*) Does not induce immunologic response.
(*b*) Does not augment immunologic response.
(6) Eliminated by host.
(7) Diluent nonsensitizing and causes no reaction.
(ii) Test Method:
(1) Application of test:
(*a*) Simple.
(*b*) Accurate dosage (amount of material/unit volume).
(2) Measurement of test:
(*a*) Round reaction.
(*b*) Clear-cut boundary.
(3) Quantitative dose response. Sufficient range of reaction size.
(4) Duration of reaction. Refined time course for the positive reaction.

The Panel also defined the characteristics of safety associated with the ideal skin test. Such a product would be documented as producing no adverse effect—immediate or long term, localized or systemic, major or minor.

Experience suggests, however, that while immediate adverse effects may become quickly apparent and easily documented, the long term impact of a subtle genetic change or a slow growing virus may not be recognized if appropriate tests are not available or are not utilized. Moreover, the human host has only a limited variety of responses by which it can evidence local damage or systemic malfunction. As a result, adverse reactions arising from a specific product may be easily but erroneously attributed to a different source, and the real correlation never identified. Thus, while by no means ideal and frequently inadequate, animal and laboratory systems are often the only available tools by which the safety of a product can be estimated.

Considering the minute quantity of material used in the skin test process and its placement in a localized area of the skin, the Panel believes that it is highly unlikely that any of the products reviewed have intrinsic mutagenic or teratogenic properties. However, the ideal test material would be shown to be free of all contaminants, including microbial agents. The test material should not be harmful to the individual.

The ideal test method would be painless, noninjurious, and bloodless.

The outline of the conceptualization of the characterizations of the ideal effective skin test continues as follows:

(b) *Safety.* (i) Product does not cause:
(1) Anaphylaxis.
(2) Severe skin reaction.
(3) Constitutional symptoms.
(4) Carcinogenic effects.
(5) Teratogenic effects.
(6) Mutagenic effects.
(7) Sensitization to product.
(8) Pain.
(9) Skin irritation.
(10) Scarring.
(11) Change in pigmentation.
(12) Reactivation of disease.
(13) Interference with action of other medications.
(ii) Test material:
(1) Free of live microbial agents (sterility):
(*a*) Bacterial.
(*b*) Viral.
(*c*) Mycoplasma.
(*d*) Fungi.
(*e*) Other.
(2) Free of other contaminants (purity):
(*a*) Microbial (nonalive).
(*b*) Nonmicrobial.
(3) Eliminated by host.
(4) Nontoxic:
(*a*) By human experience.
(*b*) By best available tests.
(iii) Test method:
(1) Painless.
(2) Noninjurious.
(3) Bloodless.

All products submitted to the Panel on Review of Skin Test Antigens were compared to this conceptual model of the ideal skin test and evaluated for both safety and effectiveness. Any variance from the ideal was thoroughly reviewed and documented.

The biological product licenses for Diphtheria Toxin for Schick Test by the Parke, Davis and Co., Lymphogranuloma Venereum Antigen by E. R. Squibb and Sons, Inc., and Tuberculin, Old, by Parke, Davis and Company are still listed as being in effect by the Bureau of Biologics as of April 29, 1977, although it is understood that the products are not being marketed. No data were submitted to this Panel for review, therefore, these products may not be determined to fall within either Category I or Category II. If products were to be marketed under these licenses, there are no data available to the Panel to indicate that the safety and effectiveness of the products and the potential benefits and risks are such that marketing should be permitted for a period of time while such data are being developed. Therefore, it is recommended that these products be placed in Category IIIB and that the licenses be placed in suspension or be revoked unless sufficient data are submitted to the Bureau of Biologics to answer the questions raised for similar products reviewed in this report.

For each product reviewed, a Generic Statement was prepared evaluating the product in general terms. This is followed by a review which evaluates each manufacturer's specific licensed biological product and concludes with the Panel's recommendations. The following Generic Statements and reviews were submitted:

COCCIDIOIDIN

GENERIC STATEMENT

The principal use of coccidioidin is as a skin test reagent to detect delayed hypersensitivity or cell mediated immunity reactions in individuals who have had immunologic experience with *Coccidioides immitis*. The coccidioidin test is not 100 percent sensitive since some infected subjects do not react to it. Both the clinical form of the disease and the time of testing after acquisition of infection are important in development of positive reactions. Most patients with nonprogressive primary coccidioidomycosis develop positive delayed skin tests within the 1st week following illness, and virtually all patients become reactive after the 3d week of illness (Ref. 2). Patients with extensive pulmonary cavitation due to coccidioidomycosis may not demonstrate positive delayed skin tests and those with severe disseminated disease are often anergic, failing to react to coccidioidin as well as to other delayed hypersensitivity intradermal skin test agents (Refs. 3 through 5). Cross reactivity is observed in some animals infected with *Nocardia asteroides, Aspergillus terreus,* and *Aspergillus fumigatus* (Ref. 6). Although coccidioidin appears to be more specific than other skin test agents such as histoplasmin and blastomycin, cross reactions with histoplasmin (Refs. 7 and 8) may be sufficiently common in clinical practice to be important.

There is considerable variation in batch to batch potency of coccidioidin but the product is apparently quite stable. The coccidioidin product when inoculated subcutaneously in Freund's complete adjuvant can sensitize rabbits. The product seems safe, and side effects have been mentioned only rarely in spite of tens of thousands of tests alluded to in various published reports. It may induce occasional uncomfortable local reactions with vesiculation, and may rarely exacerbate erythema nodosum reactions. Only two instances of anaphylaxis-like reactions have been reported (Refs. 9 and 10). The product contains thimerosal, an organic mercurial which can sensitize.

In general, coccidioidin testing is useful for epidemiologic surveys and in medical diagnosis by identifying persons with past or present infection with *C. immitis*, although negative reactions in serious systemic forms of the disease may pose a problem in clinical practice.

Coccidioidin skin test reagent is prepared from a culture filtrate of the mycelial phase of *C. immitis*, which is grown on synthetic medium containing asparagine. The major active principle of coccidioidin appears to be a glycoprotein complex with a molecular weight of 31,700 (Ref. 1). Coccidioidin is assayed in humans by comparing reactions from the newly produced product with those produced by the currently available Bureau of Biologics standard. The product is dis-

pensed in liquid form in multiple dose vials in concentrations of 1:100 and 1:10.

Coccidioides immitis, a dimorphic fungus, is usually found in lesions as a spherule and there is some evidence that a preparation similar to coccidioidin, namely "spherulin,' may ultimately provide a more sensitive skin test than coccidioidin.

PRODUCT REVIEW

The natural habitat of *C. immitis* is in the soil of an area with a hot, semi-arid environment. It is among the most virulent of the fungal pathogens. The highly endemic areas of disease include the southwestern United States and northern Mexico, with endemic foci in Central America, Venezuela, Colombia, Paraguay, and Argentina. In addition the disease has been reported to occur in Russia, central Asia, Nigeria, and Pakistan, but these reports still await confirmation (Ref. 37).

Coccidioidomycosis is usually a benign, inapparent, or mild respiratory infection which resolves rapidly. Rarely, the disease is a severe disseminating mycosis with a high fatality rate. Recovery from the mild forms of the disease usually results in lifelong immunity to reinfection. If infection is established, the disease may progress as a chronic pulmonary condition or as a systemic disease involving the meninges, bones, joints, and subcutaneous and cutaneous tissues. Such involvement is characterized by the formation of burrowing abscesses. The initial tissue response and that found in rapidly disseminating disease is suppuration. However, in established chronic and slowly advancing infection, a granulomatous reaction is found, with some areas showing a mixed-type cellular infiltrate.

Coccidioidin skin test reagent is used as a diagnostic aid based on the fact that it causes delayed hypersensitivity skin reactions in persons who have had immunologic experience with the organism. Historically, there have been scattered reports of materials used for purposes of skin testing dating to 1915, but the first substantial use of coccidioidin was in 1928 (Ref. 11). Several coccidioidin skin test preparations were developed following this, but their development was often complicated by the problem of maintaining cultures on synthetic medium for many months in order to produce potent batches (media which allow rapid growth often introduced foreign protein contaminants into the the final skin test product). This problem was ultimately solved by using a modification of the synthetic medium employed for production of tuberculin in the manufacture of coccidioidin (Ref. 3). In essence, this medium is still used by both of the current commercial producers of coccidioidin, namely Cutter Laboratories and Iatric Corporation. Initially, investigators used multiple strains of coccidioidin as the original inoculant because of possible antigenic variations. At present, the commercial producers inoculate individually and pool growths later. (The Iatric Corporation produces its skin test antigen from a supernatant of mycelial mat made from 9 different strains of the organism whereas Cutter Laboratories uses 57 strains.) The number of strains employed may not be critical, however, since there is probably little strain antigenic difference. After the sterile filtrate is diluted to desired concentration, thimerosal is added to a final 1:10,000 concentration. The standard stock solution is diluted to be bioequivalent to the reference coccidioidin (1:100). Individuals who do not respond to the 1:100 dose may be tested with the tenfold stronger concentration (1:10 dose). Individuals with considerable hypersensitivity, such as those with recent coccidioidal erythema nodosum, may be tested with dilutions higher than the 1:100 dose (e.g., 1:1000 or greater).

(a) *Effectiveness.* With regard to sensitivity (ability of the skin test material to identify all individuals with specific immunologic experience) the coccidioidin delayed skin test appears to be adequate but, as in the case of most skin tests, not ideal. It has been reported that 83 percent of patients have positive delayed skin reactions to coccidioidin during the first few days of illness, 93 percent during the 2d week, and 100 percent during the 4th week (Ref. 2). Lack of skin reactivity may be noted under the following circumstances: (1) early in the first weeks of illness, (2) dissemination of overwhelming infection, (3) in certain patients with chronic cavitary pulmonary disease, and (4) excessive time lapse between disease activity and period of skin testing. In a recent study Levine and co-workers demonstrated that failure to react to coccidioidin was noted in 35 percent of individuals who reacted to the newer spherulin skin test antigen. Also included in this study were groups of control out-patients living in non-endemic areas. None of these controls reacted to either coccidioidin or spherulin while 8.9 to 12.8 percent reacted to histoplasmin (Ref. 15).

With regard to specificity (ability to identify only individuals with specific immunologic experience) there are cross reactions with coccidioidin in patients infected with other fungi such as *Histoplasma* and *Blastomyces* species (Refs. 7, 8, 12, and 13) and possibly other unknown agents. There appear to be no significant cross reactions with coccidioidin in patients infected wih mycobacteria (Refs. 3 and 14). Cross reactions with coccidioidin in patients infected with other fungi are more common at the 1:10 dilution (Ref. 13). Reactions to coccidioidin in individuals who do not react to histoplasma seem to be rare in nonendemic areas of the U.S. and Mexico based on studies by Levine et al. (Ref. 15), Edwards and Palmer (Ref. 12), and Edwards et al. (Ref. 8). Skin reactivity to coccidioidin also appears to be reasonably specific in experimental animal models. In a study by Goodman et al. (Ref. 6), involving guinea pigs inoculated individually with 1 of 23 different fungi, most of the fungi exhibited cross reactions with histoplasmin and blastomycin in more than 10 percent of the animals, whereas only those animals infected with Nocardia asteroides, *Aspergillus terreus*, and *Aspergillus fumigatus* showed cross reactivity with coccidioidin in more than 10 percent of the animals.

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material*—(1) *Chemical composition.* The mycelial supernatant has been examined by several investigators and the contents of polysaccharide and amino acid of the protein material have been elucidated. The exact polysaccharide and protein antigenic composition is not quite clear. In a study by Pappagianis, Putnam, and Kobayashi, soluble polysaccharides from culture filtrates or mycelia of *Coccidioides immitis* consisted mainly of mannose with small amounts of galactose and another reducing sugar (Ref. 1). Ethanol precipitation yielded polysaccharide material accompanied by some nitrogenous material. The active principle of the coccidioidin appeared to be a glycoprotein complex with an average molecular weight of 31,700 (Ref. 1). There was 3 to 4 percent nitrogen present in amino acids (Refs. 1 and 17). In spite of this degree of characterization there appeared to be several antigens present in the preparation as manifested by multiple precipitin arcs on Ouchterlony double diffusion analysis. There is some indication that the total biologic activity may be due to a mixture of antigens (Ref. 16).

(2) *Reproducibility.* It has long been recognized that potency can vary from batch to batch (Ref. 3), but no variability is to be expected in lots prepared from any single batch except for human errors or perhaps deterioration of potency of the batch.

(3) *Standardization.* There appear to be no well defined laboratory systems for standardization of coccidioidin, although recent studies indicate that guinea pigs can be sensitized to coccidioidin and it should therefore be possible to use such a biological system for standardization. In general, all current standardization is performed by comparing delayed skin test reactions produced by the newly manufactured product with those produced by a Bureau of Biologics standard of coccidioidin. All of these tests are done in humans known to be coccidioidin sensitive. It is not usually clear how many individuals are tested or how their degree of immunologic experience with the organism has been verified, nor is it clear whether or not the reactions are read in a blind manner or with what dilutions they are performed.

(4) *Stability.* Coccidioidin skin test material stored at 4° C appears to be stable and uniform as indicated by repeated tests performed with lot 64D 2.5 (the Bureau of Biologics standard) over the years. Even autoclaving of coccidioidin for 30 minutes reduced the potency only slightly (Ref. 3).

(5) *Sensitization.* Studies by Wallraff and Van Liew (Refs. 18 through 20) have shown that multiple injections of coccidioidin with Freund's adjuvant into rabbits and guinea pigs could sensitize, as manifested by production of cutaneous delayed skin reactivity. In man, how-

ever, the material does not appear to be sensitizing at the recommended dosage as shown by studies of Smith in large numbers of army recruits who were repeatedly skin tested (Ref. 3). Another study by Rapaport in which 53 subjects were tested with a wide range of concentrations revealed two conversions to positive reactions (Ref. 21). Those subjects who did convert, however, had been skin tested with stronger concentrations. It is thus possible that more concentrated materials may be sensitizing. Skin test ing with coccidioidin on multiple occasions does not induce an increase in humoral antibodies to coccidioidin but serologic reactions to histoplasma antigens may be augmented (Ref. 22). A peculiar coccidioidin "re-test reaction" has been described. It is characterized by the development of an area of induration and erythema at a previously positive skin test site following a new test in a new site (Ref. 23). In another large study (Ref. 24) of laboratory workers who handled *Coccidioides immitis*, slightly over 10 percent of initial negatively reacting employees converted to positive reactions over an 18-hour observation period probably secondary to laboratory infection; significantly, many subjects in this study did not develop positive skin tests in spite of repeated skin testing with coccidioidin.

(6) *Elimination.* The method and degree of elimination of coccidioidin by the host is unknown.

(7) *Diluent.* Coccidioidin contains an organic mercurial preservative (thimerosal) in a concentration of 1:10,000 w/v (weight/volume). Thimerosal has been reported to cause skin reactions (Refs. 25 through 28). The Panel's comments regarding the sensitizing capacity of thimerosal apply to coccidioidin. See the Panel statement on "Thimerosal as a Preservative."

While it is possible that some culture media constituents in the coccidioidin preparation might sensitize, this is unlikely because the culture medium consists of relatively simply compounds in very low concentrations. The same culture medium has also been used in the preparation of tuberculin without any evidence of marked diluent reactions or sensitization. That coccidioidin per se does not cause a high incidence of irritant or allergic skin reactions is strongly suggested by several studies performed in Africa, Mexico, and the U.S.A. on thousands of subjects residing in nonendemic areas who manifested low rates of skin reactivity (in the range of 2 percent or below) to coccidioidin (Refs. 29, 15 and 12).

(ii) *Test method.* The coccidioidin skin test is a relatively simply procedure involving intracutaneous injection of 0.1 ml into the skin of the forearm. The skin test reaction boundaries are rather clear-cut and the reaction is also sufficiently reproducible to allow for determination of quantitative dose response curves (Ref. 3). The action is usually largest between 24 and 48 hours and is read at these times. An early probable irritant reaction may appear within an hour and occasionally reactions may persist for 72 hours or slightly longer (Ref. 3).

(b) *Safety.* Two episodes of anaphylaxis or anaphylactic-like reactions have been described in extremely sensitive individuals (one reported in the literature and another reported to a commercial manufacturer (Refs. 9 and 10). Erythema nodosum has been precipitated by coccidioidin skin testing (Ref. 3). Another individual with a marked local response developed late onset fever and chills which lasted for 24 hours after testing (Ref. 32). Hersh and Benson reported one immediate wheal and erythema skin reaction to coccidioidin, as did Krudy and Hoff. Similar reactions of this type have been noted by Smith and Pappagianis (Refs. 33 through 35). However, those having lengthy experience with coccidioidin indicate that constitutional symptoms of fever and malaise are rarely seen (Ref. 2). Outside of these reactions, no serious side effects have occurred in the tens of thousands of skin tests mentioned in the literature. There is no evidence for carcinogenic, teratogenic or mutagenic effects attributable to the skin test preparation. Little is known with reference to elimination of the test material by the host. The test material employed is free of live bacterial or other microbial agents. Tests are not made for viral or mycoplasma contamination and do not seem to be indicated.

The intracutaneous injection of 0.1 ml causes very little pain, infrequent bleeding, and is essentially noninjurious.

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COCCIDIOIDIN BY CUTTER

1. *Product*, Coccidioidin manufactured by Cutter Laboratories.

2. *Description.* Cutter coccidioidin is a sterile standardized liquid prepared from a pool of culture filtrates of 57 *Coccidioides immitis* isolates. It is grown on synthetic medium containing L-asparagine as described by Smith (Ref. 1). The final product contains materials released from the mycelium of all 57 isolates. It is diluted in 0.9 percent USP sodium chloride to obtain the desired concentration equivalent to the Bureau of Biologics standard (1:100 or 1:10). Thimerosal to a final concentration of 1:10,000 is added and both products are supplied in 1.0 ml vials. No stabilizers or other adjuncts are added to the product. Coccidioidin diluent (which contains 0.5 percent phenol) is also sold for those who wish to prepare higher dilutions for skin test purposes. Cutter coccidioidin is recommended as a skin test reagent for detection of delayed skin reactivity in those individuals who have had immunologic experience with *Coccidioides immitis*.

3. *Analysis*—(a) *Effectiveness.* With regard to sensitivity (identification of all individuals with specific immunologic experience), there are data available that some individuals with pulmonary disease as well as others with generalized coccidioidomycosis fail to react to coccidioidin and to other delayed skin test products (Refs. 2 through 6). Concerning specificity (ability to identify only individuals with specific immunologic experience), several studies suggest cross reactivity in patients infected with other fungi, particularly *Histoplasma capsulatum* as mentioned in the generic review (Refs. 3, and 7 through 9). Overall, there is little reason to believe that Cutter coccidioidin differs in specificity or sensitivity in any respect from that mentioned in the generic review.

(i) *Test material*—(1) *Chemical composition.* The glycoprotein and amino acid content of coccidioidin have been mentioned under "Chemical Composition" in the generic review (Refs. 10 and 11). All coccidioides isolates employed in the Cutter product were inoculated together in the initial batch 64D preparation and the final culture filtrate was prepared from a mixed mycelial growth. At present, Cutter grows 57 individual strains and combines their culture filtrates. What the contribution of any individual strain is to the final antigenic potency of the combined filtrates is not certain.

(2) *Reproducibility.* Little is known with regard to reproducibility from lot to lot. As indicated in the generic review, variation in batch potency of coccidioidin antigens has long been recognized (Ref. 1). However, lots prepared from any single batch would not be expected to vary except for human errors or batch deterioration. The manufacturer furnished several tables of reactions to simultaneous tests of two Cutter lots on five or six persons. Mean reaction diameters were very nearly the same for each of the three pairs of lots.

(3) *Stability.* Data from Cutter indicate that over a period of approximately 17 years Cutter's lot 64 bulk concentrate has deteriorated approximately 50 percent. The bulk concentrate for Cutter's new lot MO 42460 is reported to have lost approximately 40 percent of its potency in 2 years.

(4) *Standardization.* The manufacturer stated "every time a bulk product is made from the concentate, 1:10 or 1:100 dilution, a sample of each and every bulk is taken for sterility and human skin tests. This is done to ensure that the potency is correct. If the potency is correct, the lot is released for filling and finishing. The final filled, labelled lot is again submitted for clinical test and must pass the test." Cutter also states that after combining strains and mixing, small samples of various dilutions are prepared and are "submitted to a physician for human skin testing and compared to the Bureau of Biologics coccidioidin standard 64D 2.5 as well as a portion of Lot (batch) 64D 2.5 maintained at the University of California that appears to maintain greater potency than that of the Bureau of Biologics reference 64D 2.5 that had been sealed into glass ampules. By this test it is determined what dilution of the concentrated mixed antigen produces the same degree of reaction as the Bureau of Biologics reference." Additional information submitted by the manufacturer in answer to a specific question regarding standardization reveals that each new lot is tested in humans known to have a positive coccidioidin skin test, and the reactions are compared with those produced by the glass ampule-sealed U.S. Reference Standard 64D 2.5 1:100 from the Bureau of Biologics, as well as the 64D 2.5 material that has been kept in California. A table of test results is supplied for the standardization of batch MO 39406: a dilution of 1:500 was tested in three persons, 1:1000 in one person, and 1:750 in three. One of the latter group was tested with the standard on the same day. For the 1:500 dilution, eight individuals were used, and four of these had simultaneous tests with the standard. Twelve persons were tested simultaneously with the standard and a 1:400 or 1:300 dilution of the new lot. No mention is made of readings of these tests by more than one observer or of "blind" readings. Many of the measurements showed a wide variation from one right angle diameter to the other. Nevertheless, when the two diameters of each reading of the 1:400 (6 tests) and 1:300 (6 tests) dilutions of the new lot and the 1:100 standard (16 tests) are averaged, the averages of these readings are roughly comparable (26.3 mm, 30 mm, and 24.3 mm, respectively) (Ref. 27).

(5) *Sensitization.* In a study of 53 subjects in 1960 using batch 64D coccidioidin, Rappaport and co-workers found no evidence of skin sensitization in individuals tested two to four times each (Ref. 12). However, a few reactors originally negative to 1:100 coccidioidin were noted to have regained reactivity to the 1:100 coccidioidin after having been tested with the more concentrated 1:10 or 1:1 antigen. The authors note that "this appears to be similar to the findings noted in studies of tuberculin sensitivity." Thus the material does not appear to be sensitizing in man at the dosage recommended but may augment waning sensitivity. In animal studies, Smith and co-workers inoculated as much as 1 ml of coccidioidin intracutaneously and 5 ml intraperitoneally on four occasions and could not sensitize guinea pigs in this manner (Ref. 13). Other investigators using rabbits and guinea pigs showed that inoculation with immunologic adjuvants could lead to subsequent cutaneous sensitivity plus antibody production (Refs. 14 through 16).

(6) *Elimination by host.* Virtually nothing is known with regard to host elimination of Cutter coccidioidin.

The hypersensitivity reactions to thi-

(7) *Sensitizing capacity of diluent.* merosal mentioned in the Panel statement on "Thimerosal as a Preservative" might be expected with the Cutter product. In preparing greater dilutions of 1:1000 or 1:10,000 the diluent contains phenol rather than thimerosal and is less likely to cause allergic or irritant skin reactions.

(ii) *Test method.* (application, measurement and quantitation of reaction). The test is applied as a simple intracu-

[1] Cited BER (Biologics Efficacy Review) Volumes refer to submissions made by interested persons pursuant to the call for data notice published in the FEDERAL REGISTER of February 26, 1974 (39 FR 7445). The volumes will be available in accordance with the provisions of 21 CFR 601.25(b)(2) at the office of the Hearing Clerk, Food and Drug Administration, Rm. 4–65, 5600 Fishers Lane, Rockville, MD. 20857.

taneous injection of the desired dosage. The skin reaction is round and indurated with reasonably clear boundaries as stated in the generic review. There is no reason to believe that the Cutter preparation differs in any of these respects since the same test method is employed. The quantitative skin test dose response is uncertain in man as in the case of the other commercial product, but as stated in the generic review the range of reaction sizes and dosages are probably adequate for dose response curve determinations if the method proposed by Wijsmuller is employed (Ref. 17).

(b) *Safety*—(i) *Host response.* There has been extensive use of this product in each marketed dilution (1:10 and 1:100), both in civilians and U.S. armed forces. One episode of anaphylaxis to an unspecified brand of coccidioidin has been reported in the literature (Ref. 18) and another reaction of this type has been reported to the manufacturer (Ref. 19). Fever and chills beginning 8 hours after skin testing and lasting up to 24 hours were also reported in one individual who had a large local response (Ref. 20). Smith and co-workers have reported precipitation or erythema nodosum with coccidioidin (Ref. 1), but even marked reactions to coccidioidin do not evidently reactivate old infections or worsen existing ones. Hersh and Benson reported one immediate wheal and erythema reaction as did Krudy and Hoff (Refs. 21 and 22). Similar reactions of this type have been noted by Smith and Pappagianis (Ref. 23). Some of these may have been due to thimerosal sensitivity rather than coccidioidin components. Considering the very large number of skin tests performed with coccidioidin, it would appear that it is a very safe skin test agent. As an example, in over 2,000 individuals tested in one recent series (Ref. 24), 988 were reactive to coccidioidin without any marked untoward reactions. The preparation has also been used to test newborn infants and pregnant women apparently without untoward reactions (Ref. 25). The Panel found no information concerning potential carcinogenic, teratogenic or mutagenic effects from this product.

(ii) *Test material*—(1) *Sterility.* Tests for viruses and mycoplasma are not indicated and the Cutter product appears to be free of other live microbial agents and bacteria which could be identified by the routine and specific sterility tests. The product cannot be certified as free of other contaminants such as extraneous microbial protein antigen. The characteristics of the culture medium make it unlikely that other toxic substances would be carried over into the filtrate. The preparation method also makes it unlikely that non-filterable agents will be present. The filtrate from each of the 57 strains employed in preparation of Cutter coccidioidin product is tested for sterility in fluid thioglycollate medium on blood agar plates and in several mice whose lungs, livers and spleens are cultured in Sabouraud-dextrose agar with and without antibiotics at room and incubator temperatures.

(2) *Elimination by host and toxicity.* The extent and degree of Cutter coccidioidin elimination from the host is uncertain. By extensive human experience the Cutter product appears to be nontoxic.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends the product be placed in Category IIIA to remain on the market with the requirement that acceptable data based on scientifically sound methods be submitted to demonstrate efficacy in humans (Ref. 26). These data should include for each batch:

(i) Comparison of the product with the standard by means of dose response curves.

(ii) Distribution of reaction sizes in persons known to be infected and in those presumed to be uninfected with *C. immitis.*

(iii) Chemical characterization (including protein, carbohydrate, lipid and nucleic acid) should be performed to assess consistency of production.

(iv) Critical and detailed examinations of the stock strains of *C. immitis* must be made to ensure that they are not contaminated with other species of filamentous fungi whose filaments may be interlaced with those of the stock strain under the same cultural conditions (Ref. 27).

(v) Evidence should be furnished for stability of the material in the final container and the working dilution made therefrom (i.e. possible adsorption to glass or plastic).

(b) Two years is a reasonable time for the recommended testing to be accomplished.

(c) There are not sufficient data to determine if the presently distributed product is effective, i.e., the standardization of the present batch MO 39406 does not ensure that biologic activity in humans is equivalent to that of the standard.

(d) Furthermore, the Panel recommends that the following information in addition to that in the product brochure dated November 1973 should be included in the labeling:

(i) A positive reaction may cause a rise in the titer of complement fixation antibody to Histoplasma antigens, but not to coccidioidin.

(ii) A precaution against use in thimerosal sensitive subjects.

(iii) If used in individuals with erythema nodosum, the skin reaction may be violent.

(iv) Individuals infected with Histoplasma, Blastomyces, and possibly other fungi may react to this product.

(v) The product may boost the level of skin sensitivity to coccidioidin.

(vi) The skin test may be negative in severe forms of the disease or when prolonged periods of time have passed since infection.

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(28) BER Volume 5006.[1]

COCCIDIOIDIN BY IATRIC

1. *Product.* Coccidioidin manufactured by Iatric Corp.

2. *Description.* Iatric coccidioidin is a sterile culture filtrate obtained from nine selected isolates of *Coccidioides immitis*, which have been isolated in endemic areas of Arizona and California (isolates 61, 44, 14, 62, 37, 41, 60, and 43 are from the Arizona State Laboratories in Phoenix and an additional "Silvera" strain was obtained through the Naval Biological Laboratory in Oakland, California). The growth medium consists of a modification of the L-asparagine formula used to prepare tuberculin. Nine parts of this filtrate are mixed with one part of a solution containing 0.18 percent sodium bicarbonate, 0.85 percent sodium chloride, and 0.1 percent thimerosal to make the stock solution. This solution is diluted and tested in humans by comparison with the 1:100 dilution of the Bureau of Biologics coccidioidin standard for approximate equal degree of potency. This equipotent dilution is then referred to as the 1:100 dilution. The two dilutions which correspond to 1:100 and 1:10 of the standard Bureau of Biologics coccidioidin are those marketed by Iatric Corporation. One-ml vials which contain sufficient material for at least nine skin tests of 0.1-ml volume each are marketed. Iatric coccidioidin is used as a skin test antigen to detect individuals who have previous immunologic experience with *Coccidioides immitis*, via production of a delayed hypersensitivity skin response.

3. *Analysis*—(a) *Effectiveness.* In a comparison of this company's product with that of Cutter Laboratories in over 1,000 ninth grade students in San Diego, California, there were no marked differences in reactions produced by the two preparations; although it was suggested that if a negative reaction occurred with one product it "might be worthwhile to repeat the skin test with the other antigen." The manufacturer does not indicate who performed this study or whether or not it was published. It would seem difficult from the data at hand to determine what proportion of individuals who have specific immunologic experience with *Coccidioides immitis* will react with Iatric coccidioidin. The specificity of Iatric's coccidioidin has not been tested in either animal models or man.

With regard to effectiveness in animal models, there is evidently only one paper reporting the use of the Iatric preparation. Among 74 mice infected with *Coccidioides immitis*, only 20 reacted to Iatric coccidioidin 21 days following infection (Ref. 1), and only 4 of 78 animals reacted at 10 days. The other papers submitted by the manufacturer dealing with animal models and using Iatric preparations have generally related to either the effects of coccidioidin on peritoneal exudate cell migration or to studies of coccidioidin in thymectomized mice. None of these papers bear on the effectiveness of Iatric coccidioidin as a skin test reagent (Refs. 1 through 4).

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material*—(1) *Chemical composition.* The chemical composition of Iatric coccidioidin is not defined. The step by step manufacturing procedures are listed in reasonably clear fashion as mentioned earlier in the product description. There is no laboratory system used for standardization. For human testing, dilution of the filtrates from the individual strain fungal mats would appear to be the primary measurement of antigen content. No data are provided with regard to the relative contribution of the nine isolates which are used in the production of the skin test material.

(2) *Reproducibility.* There are no data directly bearing on reproducibility from lot to lot.

(3) *Standardization.* Iatric Corporation tests dilutions of the combined antigens in comparison with the Bureau of Biologics standard on more than five human subjects to determine the dilution which most closely approximates the standard (Ref. 8). The data provided by the manufacturer demonstrate remarkable agreement. Because of this unusually consistent agreement, it is unlikely that tests were performed in a scientifically acceptable manner. In fact, the agreement is closer than is normally found when the same preparation of tuberculin in the same dosage is given in different sites to the same subject (Ref. 5).

(4) *Stability.* Stability of one of their lots (No. 2AH) has been reported for samples after 1, 2, 3, and 4 years of storage tested in several individuals (a maximum of 10 and a minimum of 6 subjects) with fairly similar results. Each year the individuals were varied with some overlap. The skin test readings again showed remarkably little variability as discussed in the section on standardization.

(5) *Sensitization.* No data are provided by Iatric with regard to the ability of their specific product to augment the immune response or to sensitize after repeated injections of the recommended dosage.

(6) *Elimination by host.* Nothing is known about the product's elimination from the host outside of those reactions mentioned in the Generic Statement.

(7) *Diluent.* The hypersensitivity reactions to thimerosal mentioned in the Panel statement on "Thimerosal as a Preservative" might be expected with the Iatric product.

(ii) *Test method.* The test is administered by the Mantoux method and is presumably reasonably simple and accurate. These statements mentioned in the Generic Review should apply to the Iatric product.

(b) *Safety*—(i) *Host response.* The manufacturer claims to have no knowledge of any deleterious effects attributable to this product after having marketed hundreds of thousands of doses. No adverse reactions were described in the previously mentioned San Diego study (presumably unpublished but listed in Iatric's efficacy data report). The manufacturer provides little if any information as to safety of the product other than these anecdotal and unpublished data. The type of reaction to diluent materials, the possibility of exacerbating erythema nodosum, and the possibility of extremely rare anaphylactic reactions as previously discussed in the Generic Statement presumably apply to this product. With regard to safety data in animals, 1 ml of the Iatric preparation is injected into each of six mice and the animals sacrificed at 4 weeks. Doses of 3 ml per kilogram of body weight are inoculated into 3 rabbits. These animals are observed for any gross evidence of clinical disease over a period of 3 hours. A 0.5 ml volume of filtrate is injected into each of several mice. These animals are observed for any evidence of gross illness over a 1-week period. Five ml of filtrate are also injected into each of 2 guinea pigs which are observed over a period of 1 week. No harmful results were reported from these animal safety experiments. The Panel found no information concerning potential carcinogenic, teratogenic, or mutagenic effects from this product.

(ii) *Test material*—(1) *Sterility.* The test material is assayed for sterility in vitro in six tubes each of fluid thioglycollate medium and fluid Sabouraud medium. The cultures are incubated for a minimum of 11 days. Tests for viruses and mycoplasma are not indicated. The Iatric product appears to be free of other live microbial agents and bacteria which could be identified by the routine and specific sterility tests. The product cannot be certified as free of other contaminants such as extraneous microbial protein antigen, but the characteristics of the culture medium make it unlikely that toxic substances would be carried over into the filtrate. The preparation method also makes it unlikely that nonfilterable agents will be present.

(2) *Host elimination and toxicity.* The method and extent of elimination of Iatric coccidioidin is unknown. It would appear to be nontoxic based on limited human skin testing experience.

(3) *Test method.* The test method produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends the product be placed in Category IIIA to remain on the market with the requirement that acceptable data based on scientifically sound methods be submitted to demonstrate efficacy in humans (Ref. 6). These data should include for each batch:

(i) Comparison of the product with the standard by means of dose response curves.

(ii) Distribution of reaction sizes in persons known to be infected and in those presumed to be uninfected with *C. immitis.*

(iii) Evidence that the product is stable until the expiration date.

(iv) Chemical characterization (including protein, carbohydrate lipid and nucleic acid) should be performed to assess consistency of production.

(v) Critical and detailed examinations of the stock strains of *C. immitis* must be made to ensure that they are not contaminated with other species of filamentous fungi whose filaments may be interlaced with those of the stock strain under the same cultural conditions (Ref. 7).

(vi) Evidence should be furnished for stability of the material in the final container and the working dilution made therefrom, i.e., possible adsorption to glass or plastic.

(b) Two years is a reasonable time for the recommended testing to be accomplished.

(c) Furthermore, the Panel recommends that the following information, in addition to that in the product brochure dated February 1974, should be included in the labeling:

(i) A positive reaction may cause a rise in the titer of complement fixation antibody to Histoplasma antigens.

(ii) A precaution against use in thimerosal sensitive subjects.

(iii) The product may cause cross reactions. Individuals infected with Histoplasma, Blastomyces and possibly other fungi may react to this product.

(iv) The product may boost the level of skin sensitivity to coccidioidin.

(v) The manufacturer must modify his present statement in the second paragraph to indicate that repeated injections of 0.1 ml coccidioidin in dilutions of 1:100 have not been found to invoke the production of humoral antibodies nor induce skin sensitivity to subsequent injections.

(vi) Recommend a change in the package insert in the second sentence of the third paragraph "Negative Reaction." It should read "A negative coccidioidin reaction in the presence of erythema nodosum makes the diagnosis of coccidioidomycosis unlikely."

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(8) BER Volume 5009.[1]

HISTOPLASMIN

GENERIC STATEMENT

The term "histoplasmin" was first proposed by Zarafonetis and Lindberg in 1941 to describe two antigenic preparations from the organism *Histoplasma capsulatum* (Ref. 4). One preparation consisted of a suspension of formalin-killed whole yeast cells and the other was a cell-free culture filtrate prepared from the mycelial phase of growth. Emmons recognized the need for a standardized antigen which he produced in a modification of the synthetic medium previously recommended by Smith for the production of coccidioidin (Ref. 5). Histoplasmin H–3, made from pooled lots of two different culture filtrates of 7-months-old static growth on this medium, was tested in sensitized guinea pigs and served as the original standard skin test antigen. Realizing that the supply of this reagent would soon be depleted, Shaw et al. pooled several lots of histoplasmin, compared the pooled material's potency in sensitized guinea pigs to that of the H–3 preparation, and designated the new batch H–15 (Ref. 6). Large scale epidemiological studies soon exhausted this supply and Howell prepared yet another lot, H–42, which was tested, standardized by comparative skin tests in over 12,000 persons, and designated as the current standard skin testing histoplasmin by the Division of Biologic Standards, now the Bureau of Biologics of the Food and Drug Administration. Although a variety of preparations have been employed in the production of skin test material from the organism, the term "histoplasmin" as currently used refers to the cell-free culture filtrate obtained from the broth medium supporting the growth of the mycelial phase of *H. capsulatum* at room temperature. There is considerable variation in batch to batch antigenic composition and potency of histoplasmin despite carefully controlled conditions of growth (Refs. 12 and 13). Stock concentrates are probably stable but significant questions exist concerning the stability of diluted antigen preparations. The product seems to be safe, although all presently marketed preparations contain thimerosal, an organic mercurial which can sensitize. There is some question about the continued value of histoplasmin as a diagnostic agent, primarily because of the boost in antibody titer which may follow a positive test. This issue is discussed more completely in the detailed generic review.

PRODUCT REVIEW

Histoplasmosis is a fungus disease of man primarily involving the lungs and usually acquired by the inhalation of infectious particles of *Histoplasma capsulatum.* Although world-wide in distribution, there are certain highly endemic areas in which infection with this organism is common, although only a small proportion of those infected develop overt disease recognized as histoplasmosis. The disease is not transmitted from person to person.

Histoplasma capsulatum is a dimorphic organism. On laboratory medium incubated to 25° to 27° C it exists as the filamentous (mycelial) phase as it does in soil. In the tissues of the infected mammalian host it exists as a yeast. Various preparations of culture filtrate from each of the two phases have been used to prepare antigens for serologic analysis and for skin tests useful as aids in diagnosis.

The disease was first described in 1906 by Samuel T. Darling (Ref. 1), who considered the organism to be a new pathogenic protozoan resembling *Leishmania.* The mycotic nature of the parasite, suspected in 1912, was confirmed in 1934. Between the years 1906 and 1934 only seven human cases of histoplasmosis, all fatal, had been recorded in the literature. By 1945, the list had grown to 71 cases throughout the world, but 79 percent had occurred in the United States, mostly in residents of the Mississippi and Missouri River valleys. The concept of histoplasmosis as a rare and almost always fatal mycotic disease was shattered in 1945 by the studies of Christie and Peterson (Ref. 2) and by Palmer (Ref. 3) which demonstrated the true prevalence of histoplasmosis mainly by the use of the histoplasmin skin test reaction.

Histoplasmin is the cell-free culture filtrate obtained from a chemically defined broth medium after several months of growth of the mycelial phase of *H. capsulatum* at room temperature.

(a) *Effectiveness.* The sensitivity of the histoplasmin skin test in identification of infected individuals is relatively high. Within 3 or 4 weeks after the onset of acute or epidemic histoplasmosis the rate of positive reactions is almost 100 percent (Ref. 7). In chronic pulmonary disease, however, the rate is approximately 80 percent (Ref. 8). As in other granulomatous diseases, the disseminated form may be accompanied by a negative skin test with the homologous antigen. The skin test will be negative also in individuals who have a state of general anergy as a

result of suppressed immunity by disease or drug administration.

The reaction is not highly specific. There are extensive cross-reactions in individuals and animals sensitized to the other dimorphic fungi, especially *Blastomyces dermatitidis* and *Coccidioides immitis* (Refs. 5 and 9). Hopes for a more specific antigen have been stimulated by several investigators. Two major antigens, "h" and "m", have been purified and characterized serologically (Ref. 10). Sprouse succeeded in separating the skin test reactive materials by polyacrylamide disc electrophoresis and found a fraction which would not cross-react with *Blastomyces* (Ref. 11).

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material.* Individual lots of the test material may vary widely in antigenic composition and potency despite carefully controlled conditions of growth (Refs. 12 and 13). The antigenic composition of histoplasmin is very complex and the significance of the various antigenic fractions found in different lots of histoplasmin is poorly understood. With the agar gel immunodiffusion technique, Heiner demonstrated two significant precipitins. The "h" antigen was precipitated by an antibody associated with active infection and recent disease, whereas the "m" antigen was demonstrated by an antibody persisting from inactive disease and sometimes appearing in the anamnestic response after a positive skin test (Ref. 14). The immunogen responsible for this anamnestic response has been partially purified and characterized as the "m" reactive component. Histoplasmins deficient in the "m" antigen may not induce this humoral antibody response (Ref. 22).

The chemical composition of the skin reactive material has not been determined accurately. Some investigators considered it to be polysaccharide in nature, while others claimed that it was a protein-carbohydrate complex. Recent information indicates that the active material is either protein (Ref. 15) or complexes of protein and carbohydrate (Ref. 25).

Standardization of histoplasmin can be readily accomplished since there is a standard reference material available through the Bureau of Biologics, and there is no trouble in finding sensitized individuals. The relation between the guinea pig model and the human model needs to be established.

The stability of the stock concentrated material is good. Storage at 5° C over a period of several years does not appear to decrease its potency. The diluted antigen, on the other hand, is less stable and the loss of potency is directly related to the temperature of storage. No data are available regarding the extent of adsorption to glass or plastic and the possible influence of a stabilizing agent such as polysorbate-80. This information is badly needed.

Repeated skin tests with histoplasmin do not induce delayed hypersensitivity. Even a single positive test, however, may augment or boost the titer of certain humoral antibodies. The following is a summary of many reports that deal with this subject (Refs. 16 and 17). The appearance of significant increase of complement fixing (CF) titer to the histoplasmin antigen after a positive skin test was noted in from 3 to 58 percent of subjects. Most of the studies indicated a 20 to 30 percent rate. The maximum titers so produced varied from 1:8 to 1:256, but the usual titers were 1:8 to 1:32. Boosts to the yeast phase antigen, on the other hand, were distinctly less common, the rate varying from 0 to 14 percent. Maximum antibody levels were noted in 2 to 3 weeks following the test and they persisted for as long as 6 months. No increases were noted before the 5th day. The appearance of "m" band precipitins was noted in 12 to 90 percent of subjects after a single positive skin test.

A negative skin test very rarely causes an antibody response. A summary of 6 reports reveals that none of 313 subjects with negative skin tests had an antibody response by complement fixation to a histoplasmin antigen, and less than 1 percent of 312 subjects showed such a response to a yeast phase antigen (Refs. 17 and 18).

Questions have been raised (Ref. 19) about the advisability of continued use of histoplasmin as a diagnostic aid because of the boost in antibody titer which may follow a positive skin test (see above) and because the skin reactions to histoplasmin may not be sufficiently specific. These objections are especially applicable in areas of high endemicity where the vast majority of adult residents have positive skin tests and where serological studies would have greater diagnostic value. Conversely, there are several considerations which support the usefulness of the skin test for the evaluation of the possibility of histoplasmosis in the individual patient. The physician who uses histoplasmin for this purpose must be aware of its shortcomings. A reliable initial result can be obtained from serum taken either before or within a few days of the recognition of a positive test.

The skin test is a procedure that can be done and observed by the physician himself within 48 hours. The CF test results, on the other hand, usually involve a delay of 1 to 3 weeks because standardized antigens are not widely available in local laboratories. In areas of low endemicity it may be easier to do mass skin testing to quickly recognize outbreaks of acute histoplasmosis than to collect serum, transport it to a central laboratory, and await the CF test results.

Although histoplasmin is not as useful in diagnosis as it was once thought to be, it may serve an important purpose in the evaluation of pulmonary nodules. In areas of high endemicity a negative skin test is strong evidence against histoplasmosis, while a positive histoplasmin along with a negative tuberculin test in an area of low endemicity constitutes suggestive evidence in favor of histoplasmosis.

Finally, the considerations that determine the relative value of the skin test versus the serologic tests for diagnosis in an individual patient are those of epidemiologic and clinical nature together with the ready availability of a laboratory capable of performing accurate serologic tests for histoplasmosis.

There is no information on the rate or the mechanism of elimination by the host. Since the antigen is produced in a chemically defined medium the product should be nonsensitizing and nonreactive except for the possible influence of an added preservative.

(ii) *Test method.* The test can be administered either by the Mantoux method or by a multiple puncture tine device. The former procedure allows a measured amount of test material to be injected, the latter introduces an unknown amount. The Mantoux method is not difficult, while the multiple puncture device procedure is even simpler. In both instances, the reactions have clear-cut borders and are usually round, although the tine procedure may produce irregular reactions as a result of partial coalescence of reactions about individual puncture points. Quantitative dose response studies are possible with the Mantoux method (Ref. 23), but are difficult with the tine application because of uncertain dosage and a discontinuous distribution of reaction sizes. The reaction usually appears in 24 hours, reaches a maximum at 48 to 72 hours and slowly subsides.

(b) *Safety.* Thus far, experience with this test material reveals no safety problems in regards to anaphylaxis, constitutional symptoms, carcinogenic effects, teratogenic effects, mutagenic effects, sensitization to product, skin irritation, and reactivation of disease. Severe skin reactions are liable to occur in highly sensitive individuals just as in the tuberculin skin test.

The test material may be rendered free of live microbial agents in the usual manner and sterility tests may be performed to ensure this freedom. The method of manufacture is not likely to introduce viral or mycoplasmal agents. There is no information on the elimination of the test material by the host. There is no evidence to suggest toxicity of the test material. There should be no particular problem with the test method since it is the standard intracutaneous injection of a small amount of material. The test procedure causes very little pain or bleeding and its essentially noninjurious.

Since 1968, it has been known that mycelial fungal mats and colonies thought to be pure cultures of *H. capsulatum* may, in fact, be contaminated with fungi which are not sufficiently different in color or texture to be grossly distinguished from *H. capsulatum* (Ref. 24). The filaments of such fungi interlace with those of *H. capsulatum* in the same colony and cannot be separated except by very special mycological methods which have not been taken into account either in the preservation and maintenance of the stock cultures from which the histoplasmins were prepared or in the "purity" of the "uncontaminated" histoplasmins themselves. This omission is critical for the "histoplasmins" prepared for in-

jection by the Mantoux method but even more serious for "histoplasmins" that are concentrated 25 to 35 times and delivered much less accurately via multiple puncture devices.

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HISTOPLASMIN BY PARKE-DAVIS

1. *Product.* Histoplasmin manufactured by Parke, Davis and Co.

2. *Description.* Histoplasmin produced by Parke, Davis and Company is derived from a culture filtrate of *Histoplasma capsulatum*, strain M–1217, (PD No. 4799) originally C. W. Emmons' strain #6510. The filtrate comes from growth in a chemically defined L-asparagine broth medium after 1 to 6 months of incubation. Phosphate buffer, pH 7.4, containing sodium citrate and phenol 0.5 percent, is supplied separately as the diluent. For use as a skin test the concentrated material is diluted 1:100 with 1.0 ml of the buffer. The final diluted product contains 0.0001 percent thimerosal. A red dye (FD & C Red No. 2) at a concentration of 1:700,000 is added to assure the user that histoplasmin is present in the container.

3. *Analysis*—(a) *Effectiveness.* No specific data are furnished on the ability of Parke-Davis histoplasmin to identify all individuals who have had previous immunologic experience with *Histoplasma*. A study by Furcolow in 1950 (Ref. 1) submitted to the manufacturer demonstrated that the results with Parke-Davis lot H–11 were comparable to those obtained with the reference standard preparation H–42 in approximately 160 persons. The raw data in terms of average size of skin reaction were not made available and one cannot evaluate the conclusions of the author. New production batches and subsequent lots are standardized against U. S. Reference Standard, No. 1 by a 4x4 design on 3 to 5 guinea pigs. There is no standardization of these batches or lots in the human model. As noted in the generic review, the guinea pig model has not been correlated with the human model.

No data are provided on the specificity of the Parke-Davis product for skin testing. The generic review indicates that histoplasmin is known to be reactive in individuals infected with other fungi, especially blastomyces and coccidioides. Identity tests have been done by Parke-Davis in guinea pigs sensitized with histoplasmin, blastomycin, and tuberculin. No data are provided but the statement is made that "the homologous test antigen yielded responses equivalent to the homologous standard while the heterologus test antigens elicited little or no response." The lots of the heterologous test antigens were not noted.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material.* The product is not chemically defined but a test for identity is done by thin layer silica gel chromatography followed by ninhydrin staining. By this means it is said that the product can be distinguished from blastomycin; however, there is no evidence that this correlates with specificity in the human or animal model.

No data are presented on reproducibility from lot to lot except that the chromatographic analysis is reproducible.

The potency of each lot is estimated from a comparison of the responses obtained by the intracutaneous injection into appropriately sensitized guinea pigs of a sample of the lot under test and of the histoplasmin reference standard. The material is then diluted with phosphate buffer to give a potency of within 20 percent of that of the reference standard. No tests have been performed to correlate the guinea pig system with a system for estimation of potency in humans.

The product is stable in the undiluted form at least 1 year at 1° to 5° C.

No information was provided regarding the induction of delayed hypersensitivity or the boosting of humoral antibody titers by this product.

No data were available on elimination from the host.

The diluent contains thimerosal. The Panel's comments regarding the sensitizing capacity of thimerosal apply to this product. See the statement on "Thimerosal as a Preservative."

(ii) *Test method.* The test material is given by the simple and accurate Mantoux method.

The reactions are usually round and have a clear-cut boundary.

There is sufficient range of reaction size for dose response studies. This information comes mainly from the generic review since the only information on this subject furnished by Parke-Davis is a series of studies done by Furcolow in 1950. While no data on the actual reaction sizes were given, the proportions of patients with a positive test to three different dilutions of the Parke-Davis H–11 product indicated that quantitative dose response studies were possible.

The company recommends that the reaction be read in 24 to 48 hours.

(b) *Safety*—(i) *Host response.* There has been extensive use of this product (several million doses) with a reported minor reaction rate of 1 per 1.4 million doses sold. The nature of these reactions was not specified. Very little information is provided on the long-term safety of the red dye, FD&C Red No. 2. Except for the current suspicion about the carcinogenic potential of the dye, the Panel found no other information concerning the potential carcinogenic, teratogenic, or mutagenic effects from the Parke-Davis product.

(ii) *Test material.* By the required sterility tests, the final product contains no viable bacteria or fungi. It is not likely that other living or dead contaminants would be present, but tests for these contaminants have not been done.

The material appears to be nontoxic.

(iii) *Test method.* The test method produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends that the product be placed in Category IIIA to remain on the market with the requirement that acceptable data based on scientifically sound methods be submitted to demonstrate efficacy in humans (Ref. 2). These data should include for each batch:

(i) Comparison of the product with the standard by means of dose response curves.

(ii) Distribution of reaction sizes in persons known to be infected and in those presumed to be uninfected with *H. capsulatum.*

(iii) Chemical characterization (including protein, carbohydrate, lipid and nucleic acid) should be performed on each to assess consistency of production.

(iv) Critical and detailed examinations of the stock strains of *H. capsulatum* must be made to ensure that they are not contaminated with other species of filamentous fungi whose filaments may be interlaced with those of the stock strain under the same cultural conditions (Ref. 3).

Further data should also include:

(v) Evidence should be furnished for the long-term safety of the additive red dye or the dye should be eliminated from the product.

(vi) Evidence should be furnished for stability of the material in the final container and the working dilution made therefrom (i.e. possible adsorption to glass or plastic).

(b) Two years is a reasonable time for the recommended testing to be accomplished.

(c) The Panel also recommends that the following information, in addition to that in the product brochure dated October 1972, should be included in the labeling:

(i) Blood for serological studies (if clinically indicated) should preferably be drawn prior to administering the skin test but may be obtained within 48 to 96 hours after the test administration because a positive skin reaction may be associated with a rise in titer after that lapse of time.

(ii) It would be better to state that "the reaction should be described and measured in terms of mm of induration and degree of reaction (from slight induration to vesiculation and necrosis). In many studies reported in the literature a reaction of 5 mm or greater is considered to be positive. In case of doubt the test should be repeated but only after obtaining serum for antibody titer (if clinically indicated)."

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HISTOPLASMIN BY LEDERLE

1. *Product.* Histoplasmin (Histoplasmin Tine Test) manufactured by Lederle Laboratories.

2. *Description.* The product consists of a plastic applicator with an attached stainless steel base containing four tines approximately 2 mm in length. A preparation of stabilized histoplasmin is applied to these tines by mechanically dipping and drying. A removable plastic cap is attached and the entire applicator is sterilized by ethylene oxide gas.

The histoplasmin initially is made from three strains of *Histoplasma capsulatum* which include two soil isolates (Mexico, Mo. and Mason City, Iowa) and one human strain (A811 from the Center for Disease Control). The production medium is a semi-synthetic asparagine broth in flasks in which the strains are incubated separately 4 to 6 months at room temperature. The same number of flasks is inoculated with each strain. Each flask is examined for contamination at harvest and the contaminated flasks discarded. Equal numbers of flasks of each strain are not taken from the remaining flasks to reestablish the original exact proportion, since the inequality is slight. It is not stated how equality is determined; simply that all of the flasks with non-contaminated broth are used. The broths are then pooled, filtered, concentrated approximately 25 to 35 times, and "stabilized" with 4 percent gum arabic plus 8 percent dextrose.

In a letter of February 11, 1975, the manufacturer replied to the questions "In what way are the production culture flasks examined for identification of the organism? Was the identification macroscopic or were mycological identification techniques employed? If so, what methods were used?"

Answer. "After the culture flasks are inoculated with their respective isolates, all flasks are incubated for approximately 6 months, at which time all growth activity seems to be at a standstill. Each flask is removed from the shelf and given an identification number and is treated as a separate lot. Each flask is examined grossly, and 0.5 ml is cultured for purity on yeast agar medium plates. All flasks that do not grow out Histoplasmin [sic] capsulatum on the yeast agar plates are examined directly, and a microscopic identification of Histoplasmin [sic] capsulatum is made.

"The examination methods are microscopic and colonial, in which the morphology of the organism and the characteristic growth of the colonies are primarily used for identification. Methods used are those recommended by Professor Michael L. Furcolow, M.D., of the University of Kentucky.

"It is believed that the reason an occasional flask does not produce growth when inoculated on yeast extract agar at harvest time is that growth is inhibited by metabolic waste products that are often toxic to the culture."

3. *Analysis*—(a) *Effectiveness.* The product information supplied by Lederle contains a summary of eight published studies that compared the results of the tine test with those of the Mantoux test using commercially available histoplasmin at a dilution of 1:100. The results were variable with a reported 0 to 28 percent false negative and 0 to 13 percent false positive rate of the tine test when compared with the Mantoux test. In a later study by Perkins and Saslaw (Ref. 3) a complete correlation between the two tests was found in 34 patients with positive reactions and in 66 with negative reactions. In this study, however, hand-dipped tines were used rather than commercial tines.

No information on cross-reactions was provided.

(i) *Test material*—(1) *Chemical composition.* The chemical composition was not defined in any way.

(2) *Reproducibility.* No data were provided on the lot-to-lot reproducibility.

(3) *Standardization.* The material described as "bulk unconcentrate" is currently being standardized in guinea pigs previously sensitized with (concentrated) histoplasmin suspended in Freund's complete adjuvant. The "bulk unconcentrate" is adjusted (diluted) to compare with reactions elicited by Histoplasmin U.S. Reference, Lot No. 1, administered to guinea pigs by the Mantoux technique. The correlation of this laboratory test with a similar system in man is presented in the product information brochure in a single publication by I. R. Long of the Southern Food and Drug Research, Inc. and three other workers from Lederle Laboratories (Ref. 4). This was a report of a well-controlled comparison of test histoplasmin material with a 1:100 dilution of the Reference Standard in 200 individuals. Preliminary guinea pig tests showed that the test material was approximately three times as potent as the standard so that a 1:300 dilution of the test material was used. The reactions were read in blind fashion by four different readers. There was good correlation between positive and negative reactions using 5 mm as a criterion of positive, and between the average reaction sizes.

Standardization of the dried material on the tines, however, poses a more formidable problem. A complicated mathematical scheme is presented for estimating the potency (quantitative analysis) of the material eluted from the tines as determined by comparative Mantoux tests in sensitized guinea pigs. The object is to make the potency on each tine approximately 1/100 of that of the reference standard.

(4) *Stability.* No data were furnished on the stability of the product.

(5) *Sensitization.* It was shown by Long et al. (Ref. 4) that repeated tine tests did not induce skin sensitivity, but there is no information given regarding augmentation of the humoral antibody response. Presumably this would be the

same as or greater than that established for the intradermal test for histoplasmosis. (See the Histoplasmin Generic Review.)

(6) *Elimination by host.* There were no data on elimination by the host.

(7) *Diluent.* Reactions from the diluent may be those associated with thimerosal which varied from 1.57 to 2.4 mg/ml in the two bulk histoplasmin lots (2250–14 and HL–76) on which thimerosal assays were performed. Since 2500 tines (or 625 tine tests) require 1 ml histoplasmin, it is calculated that the tines from these lots contain 0.16 and 0.24 microgram thimerosal per tine, respectively. No thimerosal assays are done on the actual tine test units. No studies with thimerosal applied by the tine have been done in either animals or humans. The Panel's comments regarding the sensitizing capacity of thimerosal apply to this product. See the statement on "Thimerosal as a Preservative".

It was not felt that the gum arabic required for stabilizing histoplasmin for adherence to the tines would produce systemic reactions, nor that the rare local reaction which might occur (usually within one-half hour) would interfere with the reaction to the histoplasmin on the tine which is maximal at 48 or 72 hours.

(ii) *Test method.* One of the main advantages of the multiple puncture device is that its application is simple even for nonprofessional personnel and the material does not need to be refrigerated. The area of induration is usually round although partial coalescence of reactions around individual tines may produce an irregular area. None of the available reports indicates any difficulties in measurement. The range of reaction sizes is more limited than for the Mantoux technique. Inability to control the dosage and the discontinuity in the distribution of reaction sizes resulting from coalescence of reactions around individual tines make dose-response studies difficult. No information on the duration of reaction was given except the recommendation that the test be read at 48 to 72 hours.

(b) *Safety*—(i) *Host response.* Relatively limited use of this product prior to 1970 has revealed no evidence of anaphylaxis, constitutional symptoms, carcinogenic effects, teratogenic effects, mutagenic effects, sensitization to product, pain, scarring, change in pigmentation, reactivation of disease and interference with action of other medications. There were some reports in 1969 which led to the following medical advisory evaluation. "Some individuals may be extremely sensitive to the test and develop skin ulceration. Such reactions are not known to result in permanent scars."

(ii) *Test material.* Adequate sterility tests have not been done to rule out fungal contamination especially those species which produce colonies macroscopically similar to *H. capsulatum.* Viral and other nonbacterial living contaminants would not be expected. There is no information on freedom from nonliving contaminants. There is no information on elimination of the test material by the host. The material is not toxic by human experience and by the prescribed toxicity tests in two mice and two guinea pigs.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends that this product be placed in category IIIB with the license for the product to be suspended. Considerations leading to this recommendation include:

(i) The product has poor sensitivity and specificity.

(ii) There is no chemical definition of the product.

(iii) There are no data on lot-to-lot reproducibility, especially with regard to the potency of each lot of tines as they are used in humans.

(iv) There are no data on the stability of the tine material.

(v) Critical and detailed examinations of stock strains of *H. capsulatum* must be made to ensure that they are not contaminated with other species of filamentous fungi whose filaments may be interlaced with those of the stock strain under the same cultural conditions (Ref. 2).

(b) Before the license is reinstated the manufacturer must provide acceptable data based on scientifically sound methods to demonstrate:

(i) Lot-to-lot reproducibility of the final product (tine) in either a human or a human/animal model.

(ii) Stability of the final product.

(iii) Standardization in humans for future batches.

(iv) The effect of the tine skin test on the augmentation of humoral response to Histoplasma antigens and other fungal antigens.

(v) The degree of cross reactivity in geographic areas known to be highly endemic for other fungus diseases, e.g., coccidioidomycosis, paracoccidioidomycosis.

(vi) Because of the possibility that the mechanical dipping process may fail to deposit histoplasmin on the tines of individual units, routine evaluation of the final product should include a test for protein, mercury, or other test material on the tines of randomly selected individual units.

(c) Furthermore, the Panel recommends that the following revisions to the product brochure dated October 1968 and advertising copy submitted by the company should be made:

(i) Add a statement indicating that this product is not as effective as other intracutaneous histoplasmin products.

(ii) Delete the statement "should be routine for hospitalized patients."

(iii) Delete the statement "should be routine office procedure in all endemic areas."

(iv) Delete the statement "should be done concurrently with Tuberculin testing * * *."

(v) Delete the statement "should be done routinely in nonendemic areas where there is a large transient population."

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HISTOPLASMIN BY MICHIGAN DEPARTMENT OF PUBLIC HEALTH

1. *Product.* Histoplasmin manufactured by Michigan Department of Public Health.

2. *Description.* Histoplasmin produced by the Michigan Department of Public Health is a 1:400 dilution of a culture filtrate of the mycelial form of *Histoplasma capsulatum* grown for 3 months at 25° C in a chemically defined broth medium (modified Dorset-Henley liquid medium). The diluent is a glycine-glycerol-sodium chloride solution at pH 7.3 containing 0.0025 percent thimerosal. Strain 6507 (MDPH No. 1435), originally obtained from C. W. Emmons of the NIH, serves as the seed culture. The parent (batch) histoplasmin is prepared from several flasks, each containing 100 ml of medium. A batch is prepared once every 1 to 10 years from which 3 lots of 3.5 liters each are prepared annually. The final product requires no further dilution.

3. *Analysis*—(a) *Effectiveness.* The generic review provides a discussion on the sensitivity of histoplasmin. The Michigan Department of Health product was found to be comparable to the standard histoplasmin in 1953 when their lot No. 14 in a dilution of 1:400 was studied in 33 human volunteers, all of whom were positive reactors. Also submitted is an abstract of a paper in which it was reported that there was a 61 percent prevalence of histoplasmin reactivity in 1,324 children of Milan, Michigan, compared to a rate of 8.2 percent in over 7,000 children in other parts of the county. The Michigan Department of Health histoplasmin was used in that study.

No data were submitted for specificity (See Generic Review for discussion).

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material*—(1) *Chemical composition.* The chemical composition is not defined in any way.

(2) *Reproducibility.* No data were submitted on the reproducibility from lot-to-lot.

(3) *Standardization.* The Michigan material is tested for potency in guinea pigs sensitized with a mixture of "concentrated" histoplasmin together with complete Freund's adjuvant. The reactions are compared, not with the standard histoplasmin, but with "a histo-

plasmin solution equivalent in potency to 0.1 ml of a 1:100 dilution of the reference histoplasmin." Comparison of the guinea pig system to the human system is not provided directly, but lot No. 14 which was diluted to make it equally potent with the standard according to the guinea pig test was actually found to be equally potent in 24 sensitized individuals in 1953.

(4) *Stability.* No data were submitted on the stability of the product. The statement is made that "diluted histoplasmin has an expiration period of six months," but no documentation is presented. In answer to questions about stability the manufacturer states that (i) Studies done in 1954–55 in humans confirmed the stability of the final product for at least 6 months. (ii) No studies have been done to determine the amount of histoplasmin adsorbed to glass and plastic.

Further information on stability and lot-to-lot reproducibility is provided in answer to a specific inquiry. Lots 21 and 31 "in use in Michigan since 1959" were tested for potency in 4 to 6 sensitized guinea pigs, using 4 to 5 two-fold dilutions and compared with similar dilutions of reference standard histoplasmin lot No. 1. Lot 21 remained stable and comparable to the reference standard in 1959, 1962, and 1965; lot 31 was equipotent to the standard in 1968, 1971, and 1974.

(5) *Sensitization.* The sensitizing properties of histoplasmin are discussed in the generic review. The Michigan Department of Health provided no information on this subject for their product.

(6) *Elimination by host.* No data were presented on elimination from the host.

(7) *Diluent.* There was no information provided regarding the sensitizing or reaction-producing properties of the diluent except the general statement that there have been no reports of adverse reactions after the distribution of well over half a million skin test doses. The diluent contains thimerosal. The Panel's comments regarding the sensitizing capacity of thimerosal apply to this product. See the statement on "Thimerosal as a Preservative".

(ii) *Test method.* The test is given by the Mantoux method which is simple and accurate. The data indicate that a positive reaction is usually round and has a clear-cut boundary.

No data were presented about a dose response relation, but information from the generic review indicates that there is a sufficient range of reaction size with other histoplasmins.

The data presented indicate that the reaction remains for at least 72 hours.

(b) *Safety*—(i) *Host response.* No specific data were submitted regarding any short-or long-term side effects. Well over half a million doses were distributed in the state of Michigan since 1954 without any adverse reactions having been reported to the Michigan Department of Health. The Panel found no information concerning potential carcinogenic teratogenic or mutagenic effects from this product.

(ii) *Test material.* Adequate tests for absence of viable fungi were documented. Sterility tests are performed on the parent histoplasmin and on the product after final dilution. No details were given but presumably the procedures are those required by the Bureau of Biologics. Tests for other living microbial agents and other contaminants do not appear to be necessary since the product is derived from a synthetic medium.

There was no information on elimination by the host.

The product is not toxic by the human experience outlined above. The test for toxicity in animals does not meet the standards set by the Bureau of Biologics, according to the data originally submitted, but the manufacturer stated later that the safety tests do conform to the standards of the Bureau of Biologics (21 CFR 610.11). Eight mice are injected intraperitoneally with the parent material, but this is done primarily to confirm the absence of pathogenic fungi. Guinea pig inoculation is not mentioned. In Section IV, under the topic "animal safety data" (Ref. 3) reference is made to a general safety test on the finished product presumably according to regulations for the manufacture of biologic products (21 CFR 610.11).

(iii) *Test method.* The test produces very little pain and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends the product be placed in Category IIIA to remain on the market with the requirement that acceptable data based on scientifically sound methods be submitted to demonstrate efficacy in humans (Ref. 1). These data should include for each batch:

(i) Comparison of the product with the standard by means of dose response curves in humans.

(ii) Distribution of reaction sizes in persons known to be infected and in those presumed to be uninfected with *H. capsulatum.*

(iii) Chemical characterization (including protein, carbohydrate, lipids and nucleic acids) should be performed to assess consistency of production.

(iv) Critical and detailed examinations of the stock strains of *H. capsulatum* must be made to ensure that they are not contaminated with other species of filamentous fungi whose filaments may be interlaced with those of the stock strain under the same cultural conditions (Ref. 2).

In addition, the data should include evidence for stability of the material in the final container (i.e. possible adsorption to glass or plastic).

(b) Two years is a reasonable time for the recommended testing to be accomplished.

(c) Furthermore, the Panel recommends that the following information in addition to that in the product brochure dated October 1966 should be included or revised in the labeling:

(i) Blood for serological studies (if clinically indicated) should preferably be drawn prior to administering the skin test but may be obtained within 48 to 96 hours after the test interpretation (48 hours reading) because a positive skin reaction may be associated with a rise in titer after that lapse of time.

(ii) It would be better to state that "the reaction should be described and measured in terms of mm of induration and degree of reaction varying from slight induration to vesiculation and necrosis. In many studies reported in the literature a reaction of 5 mm or greater is considered to be positive. In case of doubt the test should be repeated but only after obtaining serum for antibody titer (if clinically indicated)."

(iii) Delete paragraph 2 under "RECOMMENDATIONS FOR USE" which states "the use of this product should be limited to surveys and as a diagnostic aid for those persons who have disease or X-ray findings suggestive of tuberculosis, but who are tuberculin negative."

(iv) The fourth sentence of the first paragraph under "Description of Product" should read, "As distributed, it gives skin reactions equivalent to those produced by 0.1 ml of a 1:100 dilution of the U.S. Reference Standard."

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(3) BER Volume 500t.[1]

LYMPHOGRANULOMA VENEREUM SKIN TEST ANTIGENS

GENERIC STATEMENT

Lymphogranuloma venereum (LGV) skin test antigen is prepared as an extract of embryonated chicken egg yolks infected with *Chlamydia trachomatis.* Delayed type skin reactivity appears in patients 12 to 40 days after appearance of the primary lesion and may be present for life. Skin reactions may be positive in patients with other Chlamydial infections which include trachoma, conjunctivitis, and pneumonitis.

LGV skin tests are not positive in all patients with proven immunologic experience with the Chlamydial agent or in all cases with proven lymphogranuloma venereum. A complement fixation test is available and is more sensitive than the skin test. Nonspecific reactions necessitate the use of control materials; because both the antigen and control come from living eggs that may be presumed to contain contaminating egg antigens and may also contain biologically important material not currently known or tested for. No satisfactory method of standardization exists.

PRODUCT REVIEW

Lymphogranuloma venereum is a venereal disease characterized by the appearance of a painless, small, often overlooked, genital vesicle which is followed in 1 to 2 weeks by painful swelling, and suppuration of the inguinal and femoral lymph nodes (called venereal buboes), with later production of multiple drain-

ing sinuses. From direct rectal infection coupled with the formation of scar tissue and obstruction of lymphatic channels, the complications of edema of the external genitalia, proctitis, and rectal stricture may occur years later. Disease is usually transmitted by sexual contact. The incubation period is from 7 to 14 days. The cause of the disease is *C. trachomatis*. Chlamydiae are very small microorganisms previously believed to be viruses. Their nonviral nature has been recently shown by the finding that cells possess both DNA and RNA, multiply by binary fission, possess a bacterial type of cell wall, and are inhibited in their growth by several antimicrobial agents which have no effect on viruses. Other diseases associated with *C. trachomatis* are trachoma, inclusion conjunctivitis, and mouse pneumonitis. The other species of Chlamydia, *C. psittaci*, causes ornithosis in man and many diseases in birds and mammals. Organisms of the *C. trachomatis* species are sulfonamide sensitive and possess iodine positive inclusions (glycogen-containing) whereas cells of psittaci species are sulfonamide resistant and their inclusions are iodine negative. Infections with both species of Chlamydia tend to involve both the mucous membranes of the eye and of the genitalia.

A skin test agent prepared from purulent material aspirated from unruptured LGV buboes was first employed by Frei in 1925 (Ref. 1). The pus mixed with a 4 to 6 fold excess of sterile saline was placed in a water bath at 60° C for 2 hours on 2 successive days. After examination for sterility, 0.5 percent phenol was added to the preparation as a preservative. The Frei test consists of placing 0.1 ml of the antigen into the skin of the flexor aspect of the forearm; a positive reaction was defined as a papule of at least 7mm in diameter appearing in 72 hours at the site of injection. In some cases, the papule did not appear for 5 to 8 days and it was advised that the patient be seen at intervals up to 1 week after the performance of the test. Since sufficient pus from patients was difficult to obtain, infected brains of animals were subsequently employed as antigen (Ref. 2). Because of the large percentage of false positive reactions resulting from use of infected mouse brains, chick embryo antigen prepared according to the method of Rake et al. (Ref. 3) became the most widely used skin test antigen.

The currently available antigen for lymphogranuloma venereum is prepared from Chlamydia-infected 7-day-old chick embryo yolk sacs. Pooled infected yolk sacs are blended and treated with formalin at a final concentration of 0.2 percent (v/v) to inactivate the Chlamydia. The suspension is extracted with ether for 72 hours or until a clear separation occurs between the aqueous and organic phases. The aqueous phase is removed and diluted to a final concentration of 1 percent tissue (w/v) with normal saline containing 0.1 percent formalin and 0.3 percent phenol. Uninfected yolk sacs, harvested from Salmonella pullorum-free and Rous inhibitory factor-free eggs, are extracted in a similar fashion and the preparation used as control material. Sterility and safety tests are performed on each lot of test and control material. Testing of both materials includes analytical assays for nitrogen, phenol, formalin, total solids, sodium chloride, and pH. A standard acceptable range of values is established for each determination.

All chlamydia share a common or group specific antigen which is protoplasmic, heat stable, ether soluble, trypsin resistant, and glycolipid in nature. Type specific antigen, probably a protein, is found in the cell wall. At least nine different antigenic types have been established for lymphogranuloma venereum, trachoma, inclusion conjunctivitis, and other group A organisms. Delayed type skin hypersensitivity develops in a patient 12 to 40 days after the primary lesion. Complement fixing antibodies appear at about the same time but may precede skin reactivity to the intradermal antigen. Cross reactions occur in both intradermal skin testing and in complement fixation serologic tests among patients infected with lymphogranuloma venereum and those infected with other agents belonging to the *C. trachomatis* group. The complement fixation test is generally believed to be more sensitive than the intracutaneous Frei test as a diagnostic tool (Refs. 4, 5 and 6).

Canizares reported in 1954 that repeated skin tests did not sensitize, and a test once positive remained positive for life (Ref. 7). However, the sensitivity of the skin test for detection of infected patients is difficult to determine from the literature. Older articles and books leave the impression that the Frei test is highly specific (Ref. 8 and 9) but it has been known for some time that infection with any of the Chlamydia will sensitize to the Frei antigen. Abrams in 1968 reported that 19 out of 20 patients with LGV at Letterman Army Hospital had a positive Frei test (Ref. 10). A year later, Schachter studied 12 patients mostly from the same hospital and found that only 4 out of 11 (36 percent) showed a positive Frei test while 10 out of 12 (83 percent) were positive by serologic tests (Ref. 4). Schachter also reported that the complement fixing titer was not boosted by the skin tests.

A general critique of the Frei test includes the following points: (1) It does not uniformly yield a positive reaction in all patients with clinical lymphogranuloma venereum, or in as high a percentage of patients as those with serologic evidence of having had exposure to this agent; (2) nonspecific reactions necessitate the use of control materials; (3) exposure to other agents in the Chlamydial group results in positive reactions; (4) standardization of the potency of the skin tests must be done in reactive human beings (At present there is no satisfactory experimental animal or in vitro system); (5) since the skin test antigen is made from living material, i.e. embryonated hens eggs, it is not possible at this time to be certain what agents, or other biologically important contaminants are present in the current antigen.

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LYMPHOGRANULOMA VENEREUM ANTIGEN BY LEDERLE

1. *Product.* Lymphogranuloma Venereum Antigen manufactured by Lederle Laboratories.

2. *Description.* At present, lymphogranuloma venereum (LGV) antigen is made only by Lederle Laboratories and is described in the generic review.

3. *Analysis*—(a) *Effectiveness.* The Lederle LGV Antigen does not identify all individuals who have had immunological experience with this agent. Data submitted by the manufacturer on 10 tests in 8 patients with lots 136A and 137A indicate a sensitivity of 80 percent and a specificity of 60 percent respectively. However, failure to identify people with immunologic experience has been reported to have occurred in 7 out of 10 people (Ref. 1). Extensive cross reactivity is seen in patients with other Chlamydial infections (Ref. 3 and 4).

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material*—(1) *Chemical composition.* Chemical composition is defined by nitrogen content, total solids, sodium chloride, pH, and amounts of formalin and phenol (Ref. 5).

(2) *Reproducibility.* Concerning lot-to-lot reproducibility, the following com-

parisons were made on the number of subjects indicated, according to data submitted by the manufacturer:

Current lot	Number of subjects	Mean mm of induration		Difference
		Current	Prior	
130A	5	10.8	7.8	+3.0
131A	5	7.0	(1)	
132A	3	3.7	8.3	−4.6
133A	4	8.4	7.0	+1.4
134A	3	2.7	2.7	0
135A	6	4.3	5.2	−.9
136A	6	5.2	3.5	+1.7
137A	2	0.9	2.5	+2.0

[1] Not done.

With the small number of subjects poorly documented as to disease and without information that the tests were read blindly, one can only conclude that the lots are not completely dissimilar.

(3) *Standardization.* There is no standardization in a laboratory system and no reference standard.

(4) *Stability.* There are no data on stability.

(5) *Sensitization.* Information in the literature suggests that the skin test does not induce complement fixing antibodies (Refs. 1 and 6).

(6) *Elimination by host.* No data are available on elimination of the antigen by the host.

(7) *Diluent.* There are no reports of sensitization due to phenol. Formulin, which is used in the production of the extract, has produced definite sensitization, although none from this preparation has been reported (Ref. 7).

(ii) *Test method.* (1) Application is simple and accurate, as in other "Mantoux" type intracutaneous tests.

(2) Test reaction is round and presents little problem in reading.

(3) There is a sufficient range of reaction size for quantitative dose response studies according to meager data and product information.

(4) The time of appearance of the reaction makes interpretation of one reading at a single time difficult, because some reactions may not appear for 4 or 5 days (Ref. 8), and in the earlier literature it was suggested that the patient be observed for 1 week. Insufficient numbers of known positive controls have been tested with this antigen and the test read at different time intervals from 24 hours to 1 week to determine the true time course of the reaction appearance.

(b) *Safety*—(i) *Host response.* No. adverse reactions have been reported to the manufacturer in the last 5 years. The Panel found no information concerning potential carcinogenic, teratogenic, or mutagenic effects from this product.

(ii) *Test material*—(1) Routine sterility tests are done to rule out bacterial contamination and residual viability of the Chlamydial agent. There was no information about other possible animal agents (chicken viruses) or agents which might be present in this biologic material. Rif and S. pullorom free eggs are used in the manufacturing of this product.

(2) No examinations are done for mycoplasma. No information was available to indicate whether there are microbial or nonmicrobial contaminating antigens, but by inference there are antigens derived from chicken yolk sacs present in this preparation.

(3) No information was available on the rate and method of elimination.

(4) The standard toxicity tests are performed.

(iii) *Test method.* The test produces very little pain or bleeding, and is essentially noninjurious.

4. *Recommendations.* The Panel recommends that the product should be placed in Category II as being unsafe and ineffective. The product is crude, contains a wide variety of yolk sac antigens and probably avian viruses, and does not adequately identify persons with immunologic experience with *C. trachomatis.* The following specific points apply to this product:

(a) There is poor specificity and sensitivity. There is a potentially severe penalty for lack of sensitivity in clinical applications.

(b) There is no adequate information on reproducibility.

(c) No quantitative dose/response relationship has been established.

(d) There are no data concerning the stability of the product.

(e) There is no reference material against which new lots can be standardized.

(f) There is a more sensitive serologic test.

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Mumps Skin Test Antigen

Generic Statement

The currently available skin test antigen for the detection of mumps virus hypersensitivity is a suspension of the formalin killed agent processed from the extraembryonic fluid of virus infected chick embryos. The product is marketed for use as a skin test reagent to detect delayed type hypersensitivity following an infection by the mumps virus.

Although marketed primarily to identify susceptible populations who should be protected against the disease, i.e., those in whom complications might develop (Ref. 1), it is generally employed by internists and pediatricians as one of a number of tests used to evaluate immunological (T-cell) competence in individuals with possible cell-mediated immune deficiency diseases (Ref. 2). Indeed, this latter application would appear to be one of the main current uses of the product.

Through experimental studies with monkeys, reported in 1934 by Johnson and Goodpasture, the etiologic agent of parotitis epidemica (mumps) was definitively shown to be a filterable agent (Ref. 3). It remained until later, however, for Enders and his colleagues to characterize the immune response to infection with mumps virus (Ref. 4 and 5). These early immunological studies were all performed with antigens prepared from infected monkey parotid glands (Ref. 5). Early attempts to cultivate the mumps virus in embryonated eggs were inconclusive (Refs. 6 and 7). In 1945 Habel (Ref. 8) reported cultivation of mumps virus in the developing chick embryo and demonstrated the applicability of this source of virus to complement fixation, virus neutralization and skin tests. More recently, the virus has been cultivated in tissue culture cells (Ref. 9), but this source of virus is not employed as the skin test reagent.

Heat inactivated mumps virus obtained from infected parotid glands of monkeys served as the first antigen for demonstration of skin test hypersensitivity (Ref. 5). This source of virus was soon supplanted by mumps virus propagated in embryonated chicken's egg (Ref. 8). Mumps skin test antigen is routinely available as a partially purified preparation derived from the allantoic cavity of infected chick embryos. Presently, the commercial material is an inactivated virus to which a preservative is added (Ref. 1).

Mumps virus is classified with the paramyxoviruses which form a relatively homogeneous family based on their chemical, physical and several biological properties (Ref. 10). Included with the mumps virus in this group are parainfluenza, measles, respiratory syncytial and Newcastle disease virus.

The mumps virion is characterized as a tightly coiled ribonucleic acid (RNA) nucleocapsid with helical symmetry, encased in lipoprotein envelope; glycoprotein spikes are situated on the outer surface of the envelope (Ref. 11). The virus envelope is derived from altered host cell membranes during release from the cell surface (Ref. 12). The diameters of the mumps virus particles vary between 85 and 600 nanometers with a mean of about 140 nanometers (Ref. 11).

The mumps virus possesses two major antigenic components (S-antigen and V-antigen). Antibodies produced against these antigens do not appear simultaneously (Ref. 13). The internal RNA-protein nucleocapsid represents the S-antigen and is serologically distinct from the V-antigen found in the envelope of the mumps virion (Ref. 13). It is the V-antigen which induces the formation of protective antibody (Ref. 14). In addition to a hemagglutinin, capable of agglutinating erythrocytes of many species (Ref. 15), and a weakly active neuraminidase, the mumps virus also possesses a hemolysin. While the same receptor sites on the erythrocyte appear to participate in both hemagglutination and hemolysis, the hemolysin is distinct from the hemagglutinin (Ref. 16).

The mumps virus is antigenically related to other paramyxoviruses, particularly parainfluenza type I and Newcastle disease virus (Refs. 17 and 18). Significant antigenic relationships are observed by a heterotypic antibody rise to parainfluenza virus in sera from patients with mumps (Ref. 17). Antigenic variation between strains of mumps virus has not been demonstrated (Ref. 11).

Differential centrifugation is currently the only procedure employed in the purification of mumps skin test antigen prepared from virus infected chick allantoic fluid (Ref. 1). Purification of the mumps virus by differential sedimentation may result in: (a) Large losses of virus (Ref. 19), (b) destruction of the virion (Ref. 20), or (c) co-sedimentation of normal chick embryonic particles which contaminate the final product (Ref. 21).

Employing mumps skin test antigen prepared from virus infected parotid glands of monkeys, Enders and his colleague (Ref. 5) first demonstrated a strong correlation between cutaneous reactivity and past experience with the virus. With the successful propagation of mumps virus in embryonated chicken's eggs, Habel (Ref. 8) compared the effectiveness of this source of antigen to that produced in monkeys. The relative ease of production of virus antigen by the infected chick embryo and the correlation of skin test reactivity of the in ovo derived virus to past host experience with the agent were the most important factors that resulted in the chick embyro supplanting the monkey parotid gland as the source of mumps skin test antigen (Ref. 22).

Several observations have raised serious questions concerning the specificity of the virus antigen derived from eggs. Habel (Ref. 8), in his comparative study, reported that 9 of 26 individuals who had received at least 1 inoculation of typhus fever vaccine derived from eggs gave a positive reaction to control material processed from uninfected eggs. Concern about the significant percentage of cutaneous reactivity to the avian control material alone has been voiced by several investigators (Refs. 23 through 26). In studies where avian control material has been employed, positive cutaneous reactivity to mumps skin test antigen was defined as a differential reaction between the viral material and the control of 10 mm or more (Refs. 23 through 27). When positive reactivity occurs to both virus antigen and control material, the investigator may arbitrarily eliminate or include the experimental subject as a positive reactor depending on the experimental criteria employed in the study (Refs. 24 and 25). These findings are extremely important in light of the report of St. Geme and his colleagues (Ref. 28) who observed that inoculation of inactivated mumps vaccine with two adult doses at monthly intervals into four infants under 1 year of age failed to evoke cutaneous reactivity or neutralizing antibody. Two of these infants, however, developed skin test hypersensitivity to the avian control material. These studies further emphasize that, while the currently available skin test material is simply a dilution of the mumps virus vaccine (Ref. 25), there is insufficient antigenic mass in the skin test material to induce a primary immunological response but that a secondary (anemnestic) response may occur (Ref. 28). At the present time the avian control material is commercially unavailable for routine clinical usage (Refs. 24 26).

Concern has also been raised regarding the sensitivity of the mumps skin test material derived from eggs. Employing a minimum reading of 15 mm erythema to indicate positive cutaneous reactivity to the mumps skin test antigen, Tucker and Overman (Ref. 29) documented a 25 percent false negative rate. Vicens et al. (Ref. 27) judged the skin test as only equivocally useful noting that, in their study of 133 adults, the skin test correlated only with high titers of neutralizing antibody. Over 30 percent false negative skin tests were documented by Burnell and his co-workers (Ref. 30) by a comparison of virus isolation with neutralizing antibodies and skin test reactivity in 15 children exposed to clinical mumps. These studies were confirmed by Brickman and Brunell (Ref. 24) in 59 students, demonstrating both poor correlation between skin tests and neutralizing antibodies and significant difference between two lots of skin test antigen used in the study. They experienced a 45 percent false negative rate with one of the lots compared to a 30 percent rate in the other. St. Geme (Ref. 31) confirmed and expanded on this latter finding by titrating several lots of commercial mumps skin test antigen employing hemagglutinin assay and skin test reactivity. These latter investigators demonstrated considerable variation between lots and concluded that the antigen currently available "will not allow the clinician to select with certainty the susceptible young adult who might profit from live attenuated mumps virus immunization." Brickman strengthened this conclusion (Ref. 32).

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MUMPS SKIN TEST ANTIGEN BY ELI LILLY

1. *Product.* Mumps Skin Test Antigen manufactured by Eli Lilly and Company.

2. *Description.* The mumps skin test antigen is used to evaluate immunity to mumps virus infection (Ref. 1). The mumps skin test antigen produced by Eli Lilly and Company is an eight-fold dilution, in physiological saline, of formalin inactivated vaccine (Ref. 1). For the production of mumps vaccine the virus is harvested from the extra-embryonic fluids of infected 7-day-old chick embryos, concentrated and purified by differential sedimentation, resuspended in 0.05 M glycine buffer, inactivated with formalin (1:1000), diluted with isotonic sodium chloride, and preserved with thimerosal (1:5000 ethylmercurithiosalicylate). The final mumps skin test antigen, suspended in physiological saline, contains approximately 0.006 M glycine, 1:10,000 thimerosal and less than 1:16,000 formalin.

3. *Analysis*—(a) *Effectiveness.* The ability of the mumps skin test antigen to measure the presence or absence of immunologic experience with the virus has been evaluated by the extent to which the skin test reaction correlates (i) with a history of clinical disease, (ii) with individual susceptibility as measured by subsequent infection, or (iii) with various serological indices of mumps infection. Since the original demonstration by Enders and his colleagues (Ref. 3) of the strong correlation between cutaneous reactivity to mumps skin test antigen and past host experience with the virus, several serious questions have been raised concerning the sensitivity of the commercially available skin test antigen which is derived in ovo (Ref. 4 through 13).

Enders et al. (Ref. 3), in their original studies using antigen derived from infected monkey parotid glands, correlated complement fixation titers with detailed data on the size of reactions obtained on individuals studied, and recommended that 15 mm erythema be regarded as the minimum positive reaction. They (Ref. 14) subsequently modified their criteria and concluded, from a practical point of view, that an erythematous reaction of at least 10 mm or larger be regarded as positive. Habel (Ref. 15) in his skin test study comparing mumps skin test antigen derived from chick embryo to that derived from monkey parotid glands, stated that a majority of positive cutaneous reactions (erythema) in individuals with a history of mumps was 10 mm to 20 mm in diameter. Florman and his coworkers (Ref. 16) designated 10 mm as the minimum positive reaction, whereas Tucker and Overman (Ref. 17) suggested that the 15 mm reaction be adopted to designate positive reactions. In a study with a pediatric population, Horowitz et al. (Ref. 7) employed 10 mm reaction size to designate a positive response. Brunell and his co-workers (Ref. 6) based their study on a double standard accepting a 10 mm response in children and a 15 mm reaction in adults as positive cutaneous reactivity stating that children tend to have smaller reactions to mumps skin test antigen than adults.

The available data indicate that the sensitivity of the mumps skin test antigen produced from embryonated hen's eggs by Eli Lilly and Company is variable. Vicens and his co-workers (Ref. 5) observed a 10 percent incidence of false negative skin test results in subjects who gave a positive history of mumps and they judged this test to be equivocal. Bart et al. (Ref. 12) reported that of 55 people with a positive mumps history and a positive hemagglutination inhibition serology, the mumps skin test was positive in 51 individuals.

A major criticism of most studies evaluating the specificity of the Eli Lilly and Company mumps skin test antigen has been the failure of the investigators to employ a control material prepared from uninfected extraembryonic fluids of chick embryo (Refs. 4 and 6). Eli Lilly and Company are well aware of this and state that "Pseudopositive reactions may develop in persons sensitive to egg protein" (Ref. 1), but they also state that "A control test is not necessary" (Ref. 1). Two unavoidable features of the mumps skin test antigen contribute to this property of the reagent: (a) During the maturation process of the mumps virion the lipoprotein envelope, which encases the RNA-nucleocapsid of the virus, is derived directly from the membranes of the developing chick cell (Ref. 19), and (b) in the purification procedure of the mumps virus by differential centrifugation, normal chick embryonic particles cosediment with the virus and are not removed from the final product (Ref. 20). This is further documented by data (Refs. 5, 7 through 13) indicating that the specificity of the Eli Lilly and Company mumps skin test antigen is comprised by contaminating antigens from the developing chick embryo.

There is evidence to suggest that the mumps skin test antigen may detect past host experience to other antigenically related paramyxoviruses, particularly parainfluenza type I and Newcastle disease virus (Refs. 21 and 22) Fulginiti et al. (Ref 23) support this observation, but experimental studies with monkeys failed to demonstrate an antigenic relationship between mumps virus and parainfluenza type I (Ref. 13). However, there are studies indicating that truly negative individuals are classified as positive by the skin test. Meyer et al. (Ref. 4) reported that 2 of 69 skin test positive adults subsequently developed mumps. Brunell et al. (Ref. 6) reported that 9 of 18 children who were reactive to the mumps skin test antigen subsequently developed clinical mumps.

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material*—(1) *Chemical composition.* No data are given but the major components are probably the same as those identified in the Generic Review.

(2) *Reproducibility from lot-to-lot.* Two reports deal directly with this question (Refs. 8 and 18) and the data indicate that there is wide variation in potency of different lots of mumps skin test antigen produced by Eli Lilly and Company as measured by complement-fixation antigen titration (Ref. 18) and by skin tests in humans who have neutralizing antibody (Ref. 8).

(3) *Standardization.* A direct complement-fixation test is performed on the bulk and final product employing antisera specific for mumps. The Bureau of Biologics does not have an official requirement for the Mumps virus skin test antigen, but Eli Lilly and Company has designated a minimum titer of 20 complement-fixing units per ml for each satisfactory lot with approval of the Bureau of Biologics. While Eli Lilly and Company does not state the source of their mumps specific antibody, they do require that the final product meet a minimum complement fixing standard. There is no evidence presented that indicates that the complement fixing antigen titer

correlates with cutaneous reactivity to the antigen.

There is no reference standard.

No experimental animal test model is employed that correlates to systems in humans.

(4) *Stability.* No valid data are available on this product.

(5) *Immunogenicity.* (a) The available data indicate that the mumps skin test antigen does not induce cutaneous reactivity in the skin-test-negative individual (Refs. 2 and 9). St. Geme and his co-workers (Ref. 9) demonstrated that repeated injections of the inactivated mumps vaccine, from which the mumps skin test antigen is derived, has induced cutaneous hypersensitivity of the avian antigens and egg products which contaminate the vaccine.

(b) Studies in both humans and monkeys convincingly show that the Eli Lilly and Company mumps skin test antigen contains material which may augment immunologic responses in skin test positive subjects. These augmentations in humoral antibodies may be detected as a rise in neutralizing or hemagglutination inhibition antibody titers (Refs. 6, 9 through 13 and 18).

(6) *Elimination by host.* There are no data presented regarding elimination of the mumps skin test antigen from the host.

(7) *Diluent.* The diluent contains 1:10,000 thimerosal. Eli Lilly and Company are aware of the potential sensitizing reactions that may occur to this preservative and proper instructions are given in the package insert (Ref. 1). See the Panel statement on "Thimerosal as a Preservative."

(ii) *Test method*—(1) *Application of test.* The test requires an intracutaneous injection of 0.1 ml of antigen. The procedure is simple and accurate.

(2) *Measurement of test.* (a) A review of the available literature in which the specific Eli Lilly and Company antigen was employed (Refs. 6, 7, 8, 10, 13, and 18) leads to the impression that the reaction is not round. Each of the investigators report that the largest diameter was measured and recorded.

(b) No information is given on the definition of the border of the reaction. The investigator is instructed to measure the diameter of erythema, which may be diffuse in some test subjects.

(3) *Quantitative dose response.* No data referring to possible quantitative dose response studies are available for this antigen.

(4) *Duration of reaction.* Eli Lilly and Company recommends that the test be read between 24 and 36 hours (Ref. 1). A review of the literature in which the Eli Lilly and Company mumps skin test antigen was employed reveals that the reaction can be read as early as 24 hours (Refs. 4, 6, 7, 8, 10, 13, and 18) or as late as 72 hours (Ref. 12).

(b) *Safety*—(1) *Host response.* With the exception of a reaction to thimerosal and/or to the avian antigens or egg products, there are no reports to indicate that the Eli Lilly and Company mumps skin test antigen causes anaphylaxis; severe skin reaction; constitutional symptoms; carcinogenic; teratogenic or mutagenic effects; sensitization to product; pain; skin irritation; scarring; change in pigmentation; reactivation of disease; or interference with the action of other medications.

(ii) *Test material*—(1) *Sterility.* (Freedom from live microbial agents). The bulk and final product are tested for the presence of bacteria and fungi. Specific tests for viruses and mycoplasma are not performed.

(2) *Purity.* (Free of other contaminants). No tests for purity are performed.

(3) *Elimination by host.* No data were available.

(4) *Nontoxic.* (a) Human studies suggest that the material is nontoxic (Refs. 2, 4 through 13 and 18).

(b) Animal tests required by the Bureau of Biologics are performed.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) By strict standards, this product should be placed in Category II as being ineffective for its labeled indication of identifying immunity to mumps virus infection because:

(i) There is poor specificity and sensitivity.

(ii) There is wide variation of potency from lot-to-lot as measured by complement fixation and neutralizing antibodies.

(iii) There is no uniformity in the interpretation of the test.

(iv) No quantitative dose/response relationship has been established.

(v) The material is clearly immunogenic.

(vi) There are no data concerning the stability of the product.

(vii) There is no reference material against which new lots can be standardized.

(b) However, because of its current widespread use for the nonlabeled purpose of ascertaining the immunocompetence of a patient, the Panel recommends the product be placed in Category IIIB, license to be suspended until acceptable data, based on scientifically sound methods, are submitted to demonstrate the product's safety and effectiveness in humans for an amended use. These data should include:

(i) Distribution of reaction sizes in persons known to have immune deficiencies and those presumed to be immunocompetent.

(ii) Correlation of these reactions with other in vivo and in vitro tests for immunocompetence.

(iii) Dose/response relationships should be provided and compared to a reference standard.

(iv) Evidence that the product is stable until the expiration date.

(v) Other data to indicate that the product complies with any additional requirements by the FDA for skin tests used to detect immunocompetence.

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Diphtheria Toxin For Schick Test and Schick Test Control

GENERIC STATEMENT

The Schick test reagent used to determine susceptibility to diphtheria is diphtheria toxin diluted in buffer containing a preservative (thimerosal) and a nonallergenic stabilizing agent. The potency of the Schick test dose (STD) is defined as "that amount of the standard which, when mixed with 0.001 unit of the U.S. Standard Diphtheria Antitoxin and injected intradermally in a guinea pig, will induce an erythematous reaction of 10 mm in diameter" (Ref. 1).

The test dose is introduced intracutaneously and is read at 36–72 hours. A negative test indicates that neutralization of the toxin has occurred and that diphtheria is not likely to occur in that individual on average exposure to the etiologic agent, *Corynebacterium diphtheriae*. Interpretation of a positive test is more complex in that it may indicate (a) no protective immunity or (b) hypersensitivity to the bacterial protein and/or the preservative. To distinguish between these two possibilities, a control test consisting of inactivated toxin is often used in conjunction with the diphtheria toxin for the Schick test. The Schick test control may consist of toxin heated to 65° C for 15 minutes to inactivate the toxic component or toxin inactivated by formalin.

PRODUCT REVIEW

While Klebs first discovered and described the diphtheria bacillus in 1883 (Ref. 2), it was Loeffler (Ref. 3) who reported in 1884 that in fatal cases of diphtheria, necropsy invariably revealed sterile, hemorrhagic and necrotic lesions in many organs of the body and that the causative agent, *C. diphtheriae*, could only be found in superficial membranous lesions usually localized in the throat. Four years later, Roux and Yersin (Ref. 4) reproduced these lesions in experimental animals with a potent, cell-free, heat labile toxin produced extracellularly by these organisms in the culture medium. Von Behring (Ref. 5) subsequently demonstrated that serum obtained from animals immunized against the toxin could be used in the prevention and treatment of the disease thus establishing the basis for understanding the mechanism of protective immunity to diphtheria. It remained until 1913, however, for Schick (Ref. 6) to introduce the skin test for identifying susceptibility to the toxin of *C. diphtheriae*.

The original Schick test dose (STD) was defined so that 0.1 ml was equivalent to 0.02 (1/50) minimum lethal dose (MLD) for guinea pigs. Variation in the susceptibility of the guinea pigs to the lethal activity of diphtheria toxin made it difficult to standardize the STD and for this reason the requirements for standardization of the STD were revised in the United States in 1969 (Ref. 7). The U.S. Standard Diphtheria Toxin for Schick Test is distributed by the Bureau of Biologics. It is used to determine the potency of various lots of Diphtheria Toxin for use in the Schick Test. This toxin potency is expressed in terms of the STD activity of the standard. The STD of the standard toxin is that amount which when combined with 0.001 unit of the U.S. Standard Diphtheria Antitoxin induces an erythematous reaction of 10 mm in diameter in the guinea pig. The STD of the U.S. Standard is equivalent to 1.3 International Units of the International Standard Schick Test Toxin. The U.S. Standard Diphtheria Antitoxin is also available from the Bureau of Biologics as a lyophilized preparation which has been stored at 4° C and is reconstituted to contain 6 units/ml of activity.

(a) *Attributes.* Diphtheria toxin is a protein of approximately 62,000 daltons (Ref. 8) produced only by strains of *C. diphtheriae* lysogenic for Beta-prophage carrying the *tox* gene (Ref. 9). Phage multiplication is not a necessary prerequisite for toxin production since the *tox* gene is expressed independently of the other phage genes (Ref. 10). The simple introduction of the phage genome carrying the structural gene for toxin production into sensitive strains of *C. diphtheriae* does not result in appreciable yields of toxin until the content of inorganic iron in the medium has been depleted and the concentration of iron in the bacteria has decreased to a critical level (Ref. 11). There is evidence to suggest that the repressor molecule which regulates the expression of the *tox* gene is an iron containing protein (Ref. 12). However, the mechanism by which iron mediates the effect of toxin production is not known.

The toxin molecule is released into the culture medium as a single polypeptide chain (Ref. 13), which possesses two nonoverlapping disulfide bonds (Ref. 14). It has been purified in crystalline form from culture filtrates (Ref. 15). Mild proteolytic attack with trypsin yields a molecule ("nicked protein") with two peptide fractions, A and B, still connected by the disulfide bridges (Ref. 14). Subsequent reduction and alkylation yields fragment A, the amino terminal peptide of 24,000 daltons, and fragment B, the carboxyl terminal peptide of 38,000 daltons (Ref. 13). These peptides are most effectively separated by electrophoresis or gel filtration in denaturing solvents such as 6M urea or 0.1 percent sodium dodecyl sulfate. Such treatments, however, yield B fragments which spontaneously aggregate and as a result of instability it has been difficult to biologically characterize this moiety. Fragment A on the other hand, withstands such treatment and is relatively resistant to heating and extremes of pH (Ref. 16).

Diphtheria toxin at concentration of 10^{-8}M or less was shown by Strauss and Hendee (Ref. 17) to completely block incorporation of amino acids into tissue culture cells while other metabolic activities remained normal. Collier and Pappenheimer (Ref. 18) subsequently showed that toxin, in the presence of nicotinamide adenine dinucleotid (NAD), prevented polypeptide elongation. Elongation factor (EF–2), an important component of protein synthesis, is required for translocation of polypeptidyl-transfer ribonucleic acid (RUA) from the acceptor site to the donor site on eukaryotic ribosomes (Ref. 19). EF–2 is inactivated by fragment A or diphtheria tobxin in the presence of NAD (Ref. 20). The reaction involves the transfer of adenosine diphosphate ribose (ADPribosyl) from NAD^+ to EF–2 to yield ADPribosyl–EF–2 which is inactive. The mechanism is specific for eukaryotic elongation factor and toxin has no effect on prokaryotic or mitochondrial elongation factor (EF–2) (Ref. 19). The enzymically active site occurs on fragment A but appears to be masked since the intact toxin molecule is inactive (Ref. 21).

The function of fragment B is not well characterized. It does not appear to have any enzymic activity.

Studies with various Beta-phages modified in their *tox* gene have contributed greatly to the understanding of diphtheria toxin. These mutant phages, when expressed by their host, result in the production of proteins that are not toxic or have reduced toxicity but are reactive with diphtheria antitoxin (Ref. 22). Such proteins are termed cross reactive materials (CRM). Mild proteolytic treatment with trypsin and subsequent reduction and alkylation of various purified CRMs yields fragments which may be altered in their A or B fragment (Ref. 23). Mixing and oxidizing studies using fragments obtained from nontoxic proteins have yielded fully toxic hybrid molecules (Ref. 23). The studies of Pappenheimer and his colleagues (Refs. 24 and 25) suggest that the function of fragment B is concerned with binding to specific sites on cell membranes and facilitating the entry of fragment A into sensitive cells.

(b) *Present status.* Diphtheria, until about 1885, was endemic throughout the world with epidemics and a pandemic having been recorded (Ref. 26). Between 1885 and 1941 a general decreasing trend occurred in the mortality of diphtheria with antitoxin therapy and immuniza-

tion practices contributing to this decline (Ref. 27). In the early 1940's there was a significant increase in the prevalence of diphtheria. The focus appears to have begun in Germany due in part to the internment of massive numbers of unimmunized children into concentration camps from which focus the disease spread to other parts of Europe (Ref. 28).

The incidence of diphtheria since the post World War II era has diminished to a point where the disease is considered rare (Ref. 29). This is particularly true in the age group under 10 years, owing in part to successful immunization practices. There does, however, appear to be slight increase in the incidence of diphtheria in the older age groups (Ref. 30). While the overall incidence of disease is low, it is still an important problem in areas where immunization practices are not carried out (Ref. 31).

While highly purified diphtheria toxin has been prepared in crystalline form (Ref. 15), the currently available diphtheria toxin for the Schick test is either diluted culture filtrates or one prepared by ammonium sulphate precipitation. Both methods of preparation yield reagents which contain, in addition to toxins, other growth products of the organism which may produce reactions in some individuals (Ref. 32). For this reason a Schick Test Control is often used clinically in order to interpret skin test responses. Schick Test Control material has been prepared by heat treatment to inactivate the toxic component and by formalin inactivation of toxin. The latter material is used to determine a patient's tolerance to toxoid as well as to measure his immune status.

The diluent for Diphtheria Toxin for Schick Test and the Schick Test Control contains normal human serum albumin or gelatin as a stabilizer (Ref. 33) thereby preventing rapid deterioration of the toxin. The use of human serum albumin as stabilizer has made it possible to distribute diphtheria toxin for the Schick Test in its diluted form ready for use.

While immunization with formol inactivated and alum precipitated toxoid induces protective immunity to diphtheria and has significantly reduced the mortality rate of diphtheria, there is still a need for the Schick diphtheria skin test antigen. The test can be useful in assessing the need for immunization of an individual during sporadic epidemics. It may also have a place in testing patients with suspected immune deficiencies.

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DIPHTHERIA TOXIN FOR SCHICK TEST BY MASSACHUSETTS PUBLIC HEALTH BIOLOGICAL LABORATORIES

1. *Product.* Diphtheria Toxin for Schick Test manufactured by Massachusetts Public Health Biological Laboratories (MPHBL).

2. *Description.* Schick toxin as dispensed consists of 50 Schick test doses of purified diphtheria toxin in 5 ml of sterile buffered salt solution. The toxin diluent contains 1:30,000 thimerosal as a preservative and 0.4 percent normal human serum albumin as a stabilizer. Toxin is injected intracutaneously in 0.1 ml volume and a toxoid control is injected on the opposite forearm. The reaction, if positive, appears initially at 24 to 36 hours, reaching greatest intensity by the 4th or 5th day with fading thereafter. A positive test signifies that the individual possesses little or no anti-toxin in his blood and is probably susceptible to diphtheria (Ref. 1).

3. *Analysis*—(a) *Effectiveness*—(i) *Sensitivity.*—In the diphtheria Schick test a negative response indicates immunity and a positive test susceptibility. In the efficacy data provided, a letter from a physician who evidently tested a large number of individuals, stated "on only a few occasions were positive tests found where there had been some doubt about the immunization record of the child tested" (Ref. 1). In another study by Pappenheimer, Edsall and co-workers in 1950 (Ref. 2), using the MPHBL product (Lot No. AS 51) over 2,700 soldiers were tested, 40 percent of whom were Schick positive. The manufacturer stated that based on results of this study "the test correctly defines the immune, the

nonimmune, and the hypersensitive" (Ref. 1). No specific data are provided even in the reference which they cite regarding hypersensitive responders (Ref. 2). Overall there appear to be no specific data provided in the MPHBL brochure which allow correlation of the history of previous diphtheria immunization with either a positive or negative Schick test response.

(ii) *Specificity.* Nothing is stated in the MPHBL's brochure with regard to possible cross-reactivity between their diphtheria toxin and other bacterial toxins. In animal systems the manufacturer states that "specificity is guaranteed by the fact that the minimum reaction doses of different dilutions of the parent toxin are proportional to the minimum lethal doses where lethality of the guinea pig [sic] is confirmed as diphtheritic by autopsy" (Ref. 1). An identity test is also performed on filled Schick toxin containers. In this test a mixture of product and a solution of standard antitoxin are injected intracutaneously into the guinea pig and a control containing product and saline are similarly injected: after 40 to 66 hours reactions are measured and identity and specificity are confirmed if the control shows a 20 x 20 mm reaction and the antitoxin site is free of a significant reaction. Specific data obtained with these identity tests are not provided.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material*—(1) *Defined chemical composition.* The chemical composition of MPHBL Schick test material is apparently unknown. The stock culture material is prepared from *Corynebacterium diphtheriae* Park-Williams No. 8 obtained from Connaught Laboratories. It is transplanted twice weekly to Wadsworth broth and incubated at room temperature. The production medium consists of Bacto™ casamino acids and yields toxin of 100–120 Lf (flocculation unit) per ml after 5 days incubation. This medium contains sodium chloride, cystine, CaCl2, FeSO4, maltose and NaOH. Purification of diphtheria toxin is by the ammonium sulfate method in which proteins are salted out by adding 0.45 kg of ammonium sulfate/liter of crude culture filtrate. After a process of centrifugation and reprecipitation with ammonium sulfate, the precipitate is dissolved in distilled water, dialyzed, and sterilized by aseptic filtration and addition of 1:10,000 thimerosal.

(2) *Reproducibility.* No data on reproducibility of positive reactions in man were provided. The manufacturer stated that "0.01 Lf units of toxoid are sufficient to cause a pronounced and uniform antitoxin response in persons who already possess some measure of immunity." In studies by Edsall and Wyman, duplicate Schick tests with albumin and peptone Schick toxin on 215 individuals read at 4, 7, and some cases 5 days showed only certain "minor differences" (Ref. 3). Dilution of the same stock concentrate to produce different lots of Schick toxin remained quite constant over a 9-year-period.

(3) *Standardization in laboratory systems.* Purity of the product is determined on toxin concentrates in terms of sLg per ml of nondialyzable nitrogen. The Ramon method is used for Lf and micro Kjeldahl method for nitrogen. The stock concentrate made in 1950 and still in use contains 1,300 Lf per mg of nitrogen. In preparation of the product, the manufacturer stated that "an aseptic addition is made of the same volume of a stock lot of concentrated purified toxin as was used in the last previous lot of the final product. The final toxicity is adjusted by additions of diluent or toxin as determined by prelimniary intradermal testing in guinea pigs" (Ref. 1). Data from these lot comparisons are available for lots since 1968.

(4) *Stability.* The stock concentrates of MPHBL purified diptheria toxin from which the current Schick materials are made were prepared in the year 1950 and contained 2,560 Lf and 67,000 MLD per ml. The manufacturer stated that its tests "indicate remarkable stability for this toxin concentrate, the loss in MLD being only 25 percent per decade." MPHBL diptheria toxin contains 0.4 percent normal human serum albumin as a stablizier. In studies by Edsall and Wyman in 1944, it was concluded that batches of Schick toxin stabilized with albumin showed no significant deterioration after exposure at 41° C for 24 hours (Ref. 3). The purified diphtheria toxin appears to be more stable than crude toxin and does not show the tendency toward spontaneous toxoid formation that crude preparations do, according to the studies by Pappenheimer (Ref. 4). The manufacturer holds a sample of the product at 37° C for not less than 24 hours and tests for potency. If the product passes this test it is "considered to be stable" (Ref. 1).

(5) *Sensitization.* Data were provided to show that a Schick test with its control can sensitize individuals or convert their skin test responses from positive to negative. The manufacturer believes an advantage of its product is that it uses concentrated (10 times) diphtheria toxoid as a control as opposed to unconcentrated, inactivated toxin, "because the Schick test is usually administered not only to determine the patient's immune status but also to determine his tolerance to toxoid" (Ref. 1). This amount has been shown to serve as a booster in individuals with low grade immunity but is said not to produce severe local or systemic reactions. These reactions are almost always seen in the Schick negative groups of patients who are given immunogenic doses (5 Lf) instead of skin testing doses (0.008 Lf) of toxoid.

(6) *Elimination by the host.* No data were available with regard to elimination of MPHBL diphtheria toxin by the host.

(7) *Diluent.* The diluent contains 1:30,000 thimerosal as a preservative and 0.4 percent normal human serum albumin as a stabilizer. The Panel comments regarding the sensitizing capacity of thimerosal apply to the diphtheria toxin product. (See the Panel's statement on "Thimerosal as a Preservative").

(ii) *Test method*—(1) *Application of test.* Application would appear to be simple and dosage reasonably accurate (0.1 ml volume intracutaneously, using a 27 gauge needle).

A positive test is characterized by a reaction to the Schick toxin beginning to appear in 24 to 36 hours, consisting of a circumscribed area of redness and slight induration which measures 1 cm or more in diameter. It develops gradually, reaches its greatest intensity on or about the 4th or 5th day, then fades, leaving a faint brownish pigmented spot which eventually disappears. There is no reaction at the site of the injection of the "Schick Control" (Ref. 1). A positive test signifies that the individual possesses little or no antitoxin in his blood and is probably susceptible to diphtheria.

In a positive-combined test, reactions appear at the sites of both injections, but the reaction at the site of the "Schick Toxin" injection is larger at the end of 4 days and persists longer than at the site of the "Schick Control" injection (Ref. 1). A positive-combined test indicates possible susceptibility to diphtheria and a sensitivity to the diphtheria bacillus proteins, suggesting caution in the administration of diphtheria toxoid.

The pseudo reaction is differentiated from the true positive test by the appearance of a reaction at the site of the "Schick Control" injection, and from a positive-combined test by the fact that reactions developing at the sites of both injections run a similar course, reach a maximum of intensity on the 3d day and then fade (Ref. 1). Individuals showing this reaction are hypersensitive to the diphtheria bacillus proteins and may have measurable amounts of antitoxin in their blood.

A negative reaction may be accompanied by a slight mark incident to the needle puncture, or a small area of reddening at either or both injection sites. If the test has been properly done, this negative reaction almost invariably indicates a titer of circulating serum antitoxin of at least 0.002 to .005 units per ml.

(2) *Measurement of test.* It is assumed that the test reaction is reasonably round and has clear-cut boundaries.

(3) *Quantitative dose response.* No human data were provided regarding quantitative dose response curves. In guinea pigs the manufacturer stated that intracutaneous doses larger than 0.1 ml cause severe lesions at the skin test site. Dose-response data in guinea pigs were provided for two dilutions of toxin and the test does appear to be in the appropriate range.

(4) *Duration of reaction.* The time course of a positive reaction has been stated under Paragraph 2, Description.

(b) *Safety.*—(1) *Host response.* In a study by Kuhns and Pappenheimer the MPHBL product was used in a study of immediate type skin sensitivity to diphtheria toxoid (Ref. 5). There was a high degree of correlation between personal and family history of atopic allergy and between the occurrence of immediate wheal and flare reactions to the Schick

test material. Between 1969 and 1973 the manufacturer distributed nearly one hundred thousand skin test doses. They claim that "over a 10-year-period only one report of an untoward reaction to the product" has been reported (Ref. 1.) This occurred in a group of new employees of a state school. Reactions at the sites of injections ranged from 1.5 to 2.5 cm, and painful swollen arms were reported. The manufacturer did test this same lot of toxin in 24 guinea pigs and found reactions which differed in no way from those which were typical and regularly seen with other lots of Schick test material. In a letter to the complainant, the manufacturer suggested that the procedures employed in sterilization of the test equipment might have been at fault.

No additional data were provided on animal safety other than indicating that the general safety test is performed on two mice and two guinea pigs. The Panel found no information concerning potential carcinogenic, teratogenic, or mutagenic effects attributable to this product. The test would not appear to be painful and there is no scarring except for the previously mentioned brownish discoloration after the skin testing.

(ii) *Test material.*—(1) *Sterility.* There are no routine sterility tests for viral, mycoplasma or fungal contaminants but it is unlikely that these would be present. During the manufacturing process the material is sterilized by filtration using Seitz or millipore filters after addition of diluent. This is followed by final adjustment for toxicity through addition of diluent or toxin as determined by preliminary intracutaneous testing in guinea pigs. During lot production the contents of each flask are "smeared and cultured on plain agar" and smears are stained with gram stain and examined for contaminants. On the following day, any growth on agar slants is also stained and examined.

(2) *Other contaminants.* The material contains 0.4 percent normal human serum albumin, 1:30,000 thimerosal, and buffered salt. The manufacturer stated that "absence of allergens in the medium is assured by the avoidance of macromolecular substances in the medium" (Ref. 1). The main nutrient consists of complete acid hydrolysates of casein presumably in the Bacto casamino acid. In addition the manufacturer stated that the medium is determined to be free of blood group specific substances A and B, as determined by "a method recommended by the Public Health Service" (Ref. 1). It contains less than 0.004 mg Group A substance per ml and less than 0.08 mg Group B substances per ml.

(3) *Elimination by host.* No information was available with regard to elimination of MPHBL Diphtheria Toxin by the host.

(4) *Toxicity in man and animal systems.* Toxicity in man was discussed above. Routine safety tests in mice have been carried out for 20 lots since 1953. According to the manufacturer "there has never been any noteworthy effect on these animals from the 0.5 ml injection" (Ref. 1). Guinea pigs are highly susceptible to the toxin. The administration of 5 ml results in death within 4 days due to "typical diphtherial intoxication."

Intracutaneous doses larger than 0.1 ml cause severe necrotic lesions at skin test sites and these effects are "intrinsic to the product" (Ref. 1).

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious. The time course of this reaction (maximal reactivity at 4 to 5 days with gradual fading) is however longer than the usual fungal or mycobacterial delayed type skin test reaction. The brownish pigmentation observed is also not usually recorded with other delayed skin test reagents.

4. *Recommendations.* (a) The Panel recommends that this product should be placed in category IIIA because, although it is safe, there is insufficient evidence that it is effective for its labeled use of identifying susceptibility to diphtheria. The product should remain on the market while the manufacturer supplies additional data concerning the sensitivity of the test in persons with various titers of diphtheria antitoxin.

(b) Adequate stability data should be provided. (Data are required to support the statement by the manufacturer that a product stable for 24 hours at 37° C will be stable enough to ship.)

(c) Two years is a reasonable time for the recommended testing to be accomplished.

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DIPHTHERIA TOXIN FOR SCHICK TEST AND SCHICK TEST CONTROL BY TEXAS STATE DEPARTMENT OF HEALTH

1. *Products.* Diphtheria Toxin for Schick Test and Schick Test Control manufactured by Texas State Department of Health.

2. *Description.* Texas State Department of Health (TSDH) Schick Test reagent is used to determine an individual's immunologic status in the control and prevention of diphtheria (Ref. 1). *Corynebacterium diphtheriae,* Park-Williams No. 8, isolated by the New York Bureau of Laboratories in 1894, is employed to produce TSDH Schick test diphtheria toxin. The organism was originally supplied by Connaught Laboratories (Toronto, Canada) to the Massachusetts State Department of Health who in turn provided it to TSDH. It is stored in ampules in the dried state.

The seed inoculum is initially grown in Wadsworth Proteose-Peptone broth. It is then inoculated into veal infusion broth in Povitsky bottles and incubated at 34° C for 1 week. This is followed by Seitz filtration and aging until the minimal lethal dose (MLD) has remained constant for two successive tests performed at 6-month intervals. This unpurified Schick test material is prepared for distribution in borate buffered saline solution containing 0.02 percent gelatin and 0.01 percent thimerosal. Its potency is equivalent to the U.S. standard. The potency must be such that the MLD is less than 0.0025 ml.

3. *Analysis.*—(a) *Effectiveness.* No data were provided by the manufacturer with regard to either sensitivity or specificity.

An identity test performed on filled Schick toxin containers is required by regulation. (In this test a mixture of product and a solution of standard antitoxin are injected intracutaneously into the guinea pig and the control containing product and saline are similarly injected; after 40 to 66 hours reactions are measured and identity and specificity are confirmed if the control shows a 20 x 20 mm reaction and the antitoxin site is free of a significant reaction.) This identity test is not mentioned as being performed by TSDH.

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material.*—(1) *Chemical composition.* The chemical composition of TSDH diphtheria Schick test material is apparently unknown (no data were submitted).

(2) *Reproducibility.* Regulations require each lot be standardized against the U.S. standard but no reproducibility data were submitted by TSDH.

(3) *Standardization.* No data were provided with regard to standardization of TSDH diphtheria toxin for Schick test in animal systems. The manufacturer does state that the material is tested "as per section 650.1" of Title 21, Code of Federal Regulations.

(4) *Stability.* The material contains 0.02 percent gelatin as a stabilizing agent. Regulations require that a sample of each lot be heated to 37° C for 24 hours and that its subsequent potency remain equal to that of the U.S. standard. No data are provided by the manufacturer about the stability of the product.

(5) *Sensitization.* No data were provided on the ability of repeated positive Schick tests with the TSDH product to sensitize individuals or to convert their skin test responses from positive to negative. The TSDH uses heated toxin as control material as opposed to the other producer who uses concentrated diphtheria toxoid as a control. The conditions of inactivation are not known. The man-

ufacturer states that some individuals are sensitive to diphtherial proteins other than the toxins.

(6) *Elimination by host.* No data were submitted by the manufacturer.

(7) *Diluent.* Thimerosal 1:10,000 is used as a preservative. Thimerosal has been shown to be sensitizing in some studies. The Panel's comments regarding the sensitizing capacity of thimerosal apply to this product. See the statement on "Thimerosal as a Preservative." No other data on the diluent were submitted by the manufacturer.

(ii) *Test method.*—(1) *Application of test.* The test is performed by the Mantoux intracutaneous method which is simple and reasonably accurate.

(2) *Measurement of test.* The TSDH package insert defines a positive reaction to the test as sharply defined redness 1 to 2 cm in diameter appearing in 24 to 40 hours. Nothing is said about the shape of the reaction (Ref. 1). The reaction develops gradually and reaches greatest intensity on about the 4th or 5th day followed by fading and frequent production of a faint brownish pigmented spot which eventually disappears. In this type of positive reaction there is no reactivity at the site of the Schick test control. In a so-called combined response, reactions appear at both injection sites but reactivity at the toxin site is larger at the end of 4 days. In a pseudo reaction, there is a response at the control site which runs a similar course to the response at the toxin site, both reaching a maximum intensity on day 3 followed by fading.

(3) *Quantitative does response.* No data were provided with regard to quantitative dose response curves in man.

(4) *Duration of reaction.* The TSDH Schick test reaction appears at 24 to 60 hours reaching a maximum intensity on about the 4th day and at times persisting in modified form for several weeks (Ref. 1).

(b) *Safety.*—(1) *Host response.* The manufacturer provided no information on specific adverse effects attributable to the product. In the past 10 years over 100,000 doses have been distributed and no reports of serious reactions have been reported to the TSDH. There are several published references on clinical or epidemiologic studies of diptheria in the state of Texas (Refs. 2 through 4) in which hundreds of cases were studied between 1967 and 1970. In none of these studies, however, is there any mention of use of TSDH Schick test. The Panel found no information concerning potential carcinogenic, teratogenic or mutagenic effects from this product.

(ii) *Test material.*—(1) *Sterility.* A sterility test is run on the parent material. The nature of this test was not stated but it is presumed that it consists of culturing each final lot as required by regulations.

(2) *Purity.* (other contaminants) The material contains $.02 percent gelatin as a stabilizing agent. The seed inoculum is grown in Wadsworth Proteous-Peptone broth followed by inoculation into veal infusion broth, and it is possible that both components might sensitize.

(3) *Elimination by host.* No data were provided with regard to elimination of TSDH diphtheria toxin by the host.

(4) *Toxicity in man and animal systems.* The lack of reported serious allergic or toxic reaction in man has been mentioned under "Safety." No animal safety tests were mentioned by the manufacturer.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious. The time course of the Schick reaction (maximal reactivity at 4 to 5 days with gradual fading) is longer than that observed with the usual fungal or mycobacterial delayed-type skin reaction.

4. *Recommendations.* (a) The Panel recommends that these products should be placed in Category IIIB, licenses to be suspended, because they are crude and potentially dangerous products and the manufacturer provides no data on effectiveness (sensitivity and specificity) or stability. There are no data provided confirming completion of an identity test. Before the licenses are reinstated acceptable data based on scientifically sound methods should be submitted to demonstrate effectiveness of these products in humans. Furthermore, the manufacturer should demonstrate that the veal antigens in these products do not sensitize and do not elicit false positive reactions in sensitized individauls.

(b) Two years is a reasonable time for the recommended testing to be accomplished.

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TRICHINELLA EXTRACT

GENERAL STATEMENT

The Trichinella skin test antigen is used to indicate whether or not a patient has active or "latent" healed Trichinella infection, as manifested by an immediate Type I wheal and flare reaction. The skin test becomes positive within 2 to 3 weeks after exposure and the ability to manifest a positive skin test may persist for many years. A positive skin test is, as stated by the manufacturers "only suggestive of clinical trichinosis and should be confirmed by clinical observations or muscle biopsy." The Trichinella skin test antigen does not identify all individuals with specific immunologic experience. The antigen has not been characterized, purified, or chemically defined. It is not known whether it is sensitizing in man after repeated testing or whether it stimulates production of antibody in sensitive individuals. More information is needed on the stability of standards used in quantitating and assessing biological properties of the product. There is no U.S. Reference Standard.

With regard to safety, there is anecdotal evidence that untoward reactions do not occur in man, but there are no data with regard to possible anaphylactic, carcinogenic, teratogenic, or mutagenic effects. There is a possibility of sensitization to foreign (rat) soluble protein or other impurities in the final product. Adequate sterility tests are performed for bacterial pathogens but not for viruses, mycoplasma, or fungi.

PRODUCT REVIEW

Since 1938, Trichinella extract has been prepared by the method of Bozicevich (Ref. 1). It is used in both skin and serologic tests for the diagnosis of trichinosis. The extract consists of the saline soluble constituents of dried *Trichinella spiralis* larvae harvested from muscles of laboratory infected rats by digestion in a pepsin-hydrochloric acid mixture. The rats are infected by ingesting 1 to 10 gm ground infected meat from a seed rat known to be infected with a particular strain of *T. spiralis*, and available from NIH and some health departfent laboratories. Approximately 40 days later these rats are sacrificed, skinned, and eviscerated; and their heads, legs, and tails are removed. After microscopic examination, animals found to have few or no *T. spiralis* larvae in the diaphragm (the muscle most susceptible) are discarded from the harvest pool. Muscles from all others are digested in a pepsin-hydrochloric acid mixture. The released larvae are recovered and washed 13 to 14 times in saline and finally once or twice in water to remove the saline. The larvae are stored at −70° C during the harvesting procedure, then dessicated over phosphoric anhydrate or phosphorous pentoxide.

The dried larvae are extracted with a phenolized buffered saline. Following standardization this filtered extract comprises the skin (and serologic) testing material.

Kagan and others cited by him pointed out the need for utilizing a standardized skin test procedure, including the use of a control and accurate measurement of the size of any resulting wheal (Ref. 2). A positive skin reaction is an immediate wheal and flare reaction 15 to 20 minutes after intracutaneous injection of antigen. It is said to become positive 2 to 3 weeks following infection, and persons may remain positive to the test for up to 20 years. The more usual duration of reactivity is 5 to 10 years. A delayed reaction also occurs but is much less sensitive in detecting known infections than the immediate reaction (Ref. 3). Since circulating immunoglobulin E (IgE) concentrations are increased in human trichinosis (Ref. 4), they probably play an important role in cutaneous reactivity. However, various immunoglobulins from trichinosis patients possess antibody activity to trichinella antigens (Ref. 5).

The skin test material is obviously crude. As a diagnostic tool, the skin test has largely been supplanted by serologic tests such as bentonite flocculation (BF) and charcoal particle flocculation or agglutination. The latter test can be used in the field. A positive test provides the same diagnostic information as a skin test and does not require the intracutaneous introduction of a very crude product which by its very nature must contain soluble rat proteins and possibly also rodent viruses and mycoplasmas. The product is not tested for these potential contaminants prior to use in human subjects.

(a) *Effectiveness*—(1) *Sensitivity*. The major criticism of the skin test has been that it is much less sensitive in detecting early acute infections than the BF test, other flocculation tests, and the immunofluorescent antibody (IFA) test. Humoral antibodies that react in the BF test recede much more rapidly than reactions to the skin test. For this reason, Kagan noted that the skin test may be a useful epidemiologic tool in surveying the prevalence of human trichinosis in endemic areas while serologic tests are preferred for clinical diagnosis (Ref. 2). It is recognized that a positive skin or serologic test result on an individual in an endemic population may still need to be confirmed by muscle biopsy.

Zimmerman et al. estimated the prevalence rate of trichiniasis in the human population of the U.S. as falling between 4 and 6 percent (Ref. 6). Kagan also stated that commercial skin test antigens of low protein nitrogen content lacked sensitivity compared to more potent experimental skin test antigens (Ref. 2). The commercially available skin test antigens have also been found to be less sensitive than the BF and IFA tests (Refs. 7 and 8). However, Ward et al. found no differences between the results of the BF test and a commercial skin test in diagnosing human trichinosis (Ref. 9).

(2) *Specificity*. The skin test material is not specific for *T. spiralis* infection. It cross-reacts with *T. trichuria* as well as other helminths. Parasites causing heterologous reactions include *Ascaris lumbricoides, Toxocara canis* (Ref. 10) and possibly *Echinococcus* species, *filariae*, and *schistosomes* (Refs. 11 and 12). The test does not differentiate between latent and active infections.

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TRICHINELLA EXTRACT BY LEDERLE

1. *Product*. Trichinella Extract manufactured by Lederle Laboratories.

2. *Description*. The Lederle product is a saline soluble extract of dried *Trichinella spiralis* larvae prepared as described in the Generic Statement.

3. *Analysis*—(a) *Effectiveness*. Skin tests with the Lederle Antigen may be negative in patients with *T. spiralis* infections (Ref. 1). The test may also be positive in infected individuals who do not develop overt disease (Ref. 2). Other comments in the Generic Statement generally apply to the Lederle product.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material*—(1) *Chemical composition*. The chemical composition was not defined.

(2) *Reproducibility from lot-to-lot*. No data were available except as mentioned under "Standardization" below.

(3) *Standardization*. The tests to assess the potency of each batch of skin testing product include: (a) Ouchterlony double diffusion versus antisera prepared in rabbits or deep frozen sera from confirmed cases with a previously standardized extract as control, (b) Oudin's single immunodiffusion technique to determine the amount of active material present. Antigens identified by these techniques do not necessarily relate to those concerned with the "allergenicity" of the test material. Finally, (c) the Prausnitz-Kusner reaction is applied in healthy human volunteers to further assess the potency of the product.

(4) *Stability*. No data were available.

(5) *Sensitization*. Repeated application of the skin test material may induce a delayed type skin reaction in noninfected guinea pigs, but not the immediate wheal and flare reaction. Nothing is known of this capacity in human subjects. Kim et al. observed that guinea pigs could be sensitized by multiple inoculations of prepared trichinella antigens (Ref. 3). Sensitization occurs more readily when Freund's adjuvant is also administered. There is no evidence concerning the possible sensitizing effect of the skin test antigen in humans.

(6) *Elimination by host*. No data were available.

(7) *Diluent*. The usual diluent contains phenol as a preservative, which is not sensitizing.

(ii) *Test method*—(1) *Application*. The test is administered by the Mantoux method, which is simple and reasonably accurate.

(2) *Measurement*. The reaction is irregular and difficult to measure.

(3) *Quantitative dose response*. Dose response studies are possible but are difficult owing to the irregularities of the reactions.

(4) *Duration*. An advantage of this test is that it may be read within 15 to 20 minutes after administration.

(b) *Safety*—(i) *Host response*. There is no evidence that this material causes severe skin reactions, constitutional symptoms, pain, skin irritation, scarring, pigmentation, reactivation of disease or interference with other medications. Because of its animal nature, sensitization and anaphylaxis are possible side effects. The Panel found no information concerning carcinogenic, teratogenic or mutagenic effects from this product.

(ii) *Test material*—(1) *Sterility*. The product is free of bacterial and fungal contaminants, but viruses could be present.

(2) *Purity*. The material undoubtedly contains rat proteins or their break down products.

(3) *Elimination by host*. No data were available.

(4) *Toxicity*. No data were available except isolated experiences with this material in human testing. The standard general safety tests are applied.

(iii) *Test method*. This test produces very little pain or bleeding and is essentially noninjurious except for the potential risk of sensitization or anaphylaxis.

4. *Recommendations*. The Panel recommends that this product be placed in Category II, unsafe and ineffective with the license for the product to be revoked.

(a) The safety considerations leading to this recomendation include:

(i) The product is likely to contain rat proteins.

(ii) Rat virus material is a potential contaminant.

(iii) The product might contain antigenic material from other parasites which may be found in rats.

(iv) The material has the potential for sensitization.

(v) The recommended dosage volume is hazardous when administered without a prior low volume sensitivity test such as a scratch test.

(vi) A safe serologic test exists.

(b) The product is judged to be ineffective as a diagnostic test:

(i) It has poor sensitivity in that it does not adequately identify individuals with specific immunologic experience.

(ii) It presumably lacks specificity in humans since cross reactions have been demonstrated to occur in animals infected with other helminths.

(iii) The product is poorly standardized.

(iv) The antigen has not been characterized, purified or chemically defined.

(v) There is no documentation of product stability.

(vi) A serologic test is available as an alternate diagnostic tool.

(c) Any person preparing this product in the future should be encouraged to isolate and purify the major active component demonstrable in immunodiffusion tests.

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TUBERCULIN, OLD

GENERIC STATEMENT

Old Tuberculin (OT) is a diagnostic skin test reagent prepared from filtrates of sterilized cultures of tubercle bacilli. It is used primarily to detect delayed hypersensitivity in individuals who have had immunologic experience with *Mycobacterium tuberculosis or Mycobacterium bovis*.

Originally produced by Koch in the late nineteenth century, the term "tuberculin" was later extended to a wide variety of preparations capable of eliciting the reactions of delayed hypersensitivity in a sensitized host. At the present time there is considerable variation in production methods and medium constituents for OT.

A positive tuberculin test usually develops 2 to 10 weeks after initial infection with *M. tuberculosis* or *M. bovis*. Old Tuberculin possesses excellent, although not perfect, ability to identify all individuals with this specific immunologic experience. Although sensitivity tends to persist throughout life, it may wane or disappear under a variety of circumstances. When the usual causes for negative reactions are absent, failure to react to a tuberculin test is helpful in excluding the diagnosis of tuberculosis. Skin reactions to OT may occur in individuals infected with mycobacteria other than *M. tuberculosis* or *M. bovis*.

Most of the valid data on sensitivity and specificity of tuberculin have been obtained from studies using Tuberculin, purified protein derivative (PPD).

Old Tuberculin does not have a simple definable chemical composition. Variation in potency from batch to batch is almost certain to occur. Although it should be possible to standardize OT, there is no evidence that adequate current standardization methods have been employed for batch to batch or lot-to-lot equivalence.

Old Tuberculin is applied by the intracutaneous (Mantoux) or multiple puncture techniques. The Mantoux test is simple and accurate. Multiple puncture tests are simple but deliver a variable and undetermined quantity of test material into the skin.

Old Tuberculin appears to be stable under ordinary conditions of storage and has had widespread usage in millions of persons over many years, with great safety.

PRODUCT REVIEW

Old Tuberculin is a filtrate prepared from sterilized concentrated broth cultures of tubercle bacilli, diluted with saline/glycerine and preserved with phenol. It was first produced in the late nineteenth century by Robert Koch (Ref. 1). It has been used primarily as a diagnostic skin test reagent to detect delayed hypersensitivity in individuals who have had immunologic experience with *Mycobacterium tuberculosis* or *Mycobacterium bovis*.

Shortly after he discovered the tubercle bacillus, Koch recognized the biologic activity of the products of the organism (Ref. 2). Although his dream of usinging "tuberculin" as a therapeutic agent in tuberculosis was soon dispelled, the material subsequently proved to be an aid in defining and understanding the immunology and pathogenesis of tuberculosis and in elucidating the mechanisms of delayed hypersensitivity and cellular immunity in general.

The name "tuberculin" was originally used by Koch to designate the active material in the liquid medium in which tubercle bacilli had grown. The use of the term was later extended to a wide variety of preparations capable of eliciting the reactions of delayed hypersensitivity in a sensitized host (Ref. 3), Koch produced his tuberculin by growing tubercle bacilli in glycerinated meat broth for 6 to 8 weeks at 37° C, boiling to kill the organisms, evaporating the medium to one-tenth of the original volume, and filtering to eliminate the bacilli. The finished product contained about 40 percent glycerin and it was later called "Old Tuberculin". The glycerinated meat broth was later replaced by a synthetic medium consisting of dipotassium phosphate, sodium citrate, magnesium sulfate, ferric citrate, asparagine, glucose, and 10 percent gylcerin. The first standardized Old Tuberculin in this country was produced by the Bureau of Animal Industry using this synthetic medium, and the product was called HCSM tuberculin (heat concentrated synthetic medium). Other changes in the method of manufacture of this product included the use of flowing steam for sterilization instead of boiling, growth of the organisms as a pellicle on the surface, the use of gauze for preliminary filtration of the medium with its mat of growth, the further addition of glycerin, and concentration by evaporation followed by dilution with a weak solution of phenol. At the present time there is considerable variation in production methods and medium constituents.

Injection of tuberculin into the skin or its application to the skin surface produces a localized reaction in sensitized persons. At the present time in the United States, OT is administered intracutaneously by the Mantoux (injection) method and by multiple puncture devices. The reaction to injected tuberculin in sensitized individuals consists of an area of induration (usually with surrounding erythema) which varies in size and intensity according to the dosage of tuberculin and the sensitivity of the individual. Skin reactions to OT may occur in individuals infected with mycobacteria other than *M. tuberculosis* or *M. bovis*.

A positive tuberculin test usually develops 2 to 10 weeks after initial infection with *M. tuberculosis* or *M. bovis*. Once acquired, sensitivity to tuberculin tends to persist throughout life. It may wane with advancing age or disappear if treatment of the infection is given in its earliest stages. Sensitivity may decrease or disappear temporarily during a variety of intercurrent illnesses, during treatment with adrenal corticosteroids, and in tuberculosis patients who are very sick (Ref. 4). When the usual causes for negative reactions are absent, failure to react to a tuberculin test is helpful in excluding the diagnosis of tuberculosis.

(a) *Effectiveness.* Most of the valid data on sensitivity and specificity of tuberculin have been obtained from studies using Tuberculin, PPD (purified protein derivative). Sensitivity and specificity of OT may be affected by variations in preparation. There are also problems of standardization, arising particularly from the fact that preparations found to be equivalent in guinea pigs may not be equivalent in humans (Refs. 5 through 7). The known propensity of Tuberculin, PPD to adhere to glass or plastic surfaces (Refs. 8 and 9) raises questions about the likelihood of adsorption of protein from OT as well.

(1) *Sensitivity.* Tuberculin possesses excellent, although not perfect, ability to identify all individuals with specific immunologic experience. Virtually all tuberculosis patients will react to the intracutaneous injection of tuberculin if a sufficiently strong dose is used (Refs. 10 through 14).

The intensity of the reaction may be diminished by many factors which affect delayed hypersensitivity reactions in a nonspecific manner by mechanisms not fully understood. These factors or conditions include acute virus infections or vaccination with live virus vaccines; immunosuppression by disease, drugs, or steroid hormones; a state of general anergy such as that associated with sarcoidosis or malignancy, especially lymphoma; malnutrition, especially in children; overwhelming infection; and the waning of delayed hypersensitivity associated with advancing age (Refs. 15 through 22). In addition, there is a small proportion of individuals with tuberculosis who have none of the above conditions but who still do not react to ordinary doses of tuberculin. Although a recent publication suggests that this proportion may be as high as 20 percent

(Ref. 23), observations over many years have documented that it is usually no more than 5 percent (occasionally up to 10 percent) with the use of intermediate strength tuberculin (5 tuberculin units (TU)) and 1 percent or less with stronger (100 to 250 TU) doses of tuberculin (Refs. 10 through 13). A negative tuberculin test in patients with serious or widespread disease will usually become positive after a few weeks of treatment.

(2) *Specificity.* Although the tuberculin test is very good for the recognition of tuberculous infection, infections with mycobacteria other than *M. tuberculosis* or *M. bovis* can cause reactions to tuberculin (Refs. 24 through 26). This is not surprising since all mycobacteria and many nocardia species share antigens (Refs. 27 through 32). The specificity of Tuberculin, PPD test varies geographically according to the prevalence of these other infections, specificity generally being highest in the Arctic and at high altitudes and lowest in the tropics and at low elevations (Ref. 33). Presumably, a geographic variation in specificity similar to that shown for Tuberculin, PPD also applies to OT.

Specifically is intimately related to the concentration of tuberculin used for the test. A larger proportion of persons will react to high dose tuberculin than to low or intermediate dose tuberculin. Some of these individuals have been infected with *M. tuberculosis*, some with other species of mycobacteria, and some probably with other organisms that share antigens in the cell wall, such as nocardia and corynebacteria. The main value of a high-dose tuberculin test is in a negative reaction, which makes a diagnosis of mycobacterial infection unlikely in the absence of the conditions listed above which diminish all delayed hypersensitivity reactions.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material*—(1) *Chemical composition.* Extensive investigation has resulted in a wealth of information on the chemical composition of Old Tuberculin. However, it is obvious from its preparation that it is unlikely to have a simple definable chemical composition. Long's 1939 statement (Ref. 34) probably holds true today: "Old Tuberculin is not a unit substance, but a mixture of extreme complexity. Its potency is due to about one percent of its total substance. Ninety-nine parts out of a hundred are inert. Whether, in the ninety-nine parts, there are elements present capable of eliciting nonspecific reactions, is debatable, but obviously the absolute specificity of a tuberculin containing protein derivatives from beef- or milk-peptone cannot be guaranteed. Moreover, in all probability, the actual content of active principle is never twice the same."

(2) *Reproducibility.* It has long been recognized that potency can vary from batch to batch. Variation is almost certain to occur because of variations in bacterial growth, but is difficult to assess because of poor standardization. Seibert made six batches of Tuberculin, PPD from OT in which the yield of PPD varied from 9.8 to 22.8 gm per liter of the original tuberculin filtrates (Ref. 35).

(3) *Standardization.* Following batch standardization in human populations, it should be possible to standardize OT lots against a standard preparation, using sensitized guinea pigs, provided adequate numbers of animals are used and readings are made in a fashion that obviates reader bias (Ref. 36). No evidence is provided that current standardization methods for OT employ these procedures. Standardization in an animal model may not reflect the situation in other animals or humans (Refs. 5 through 7). E. R. Long stated 35 years ago, "It is true and has been known for years that the various preparations of Old Tuberculin on the market vary greatly in their capacity to elicit reactions" (Ref. 34). This statement probably holds true now.

(4) *Stability.* Although inadequacies of standardization impede testing for stability, it is generally believed that concentrated OT is very stable under ordinary conditions of storage. Indirect evidence for stability is the fact that some concentrates from which potent OT has been made were prepared decades ago (Ref. 37).

(5) *Sensitization.* No data were found on the ability of OT to induce or augment an immunologic response.

(6) *Elimination by host.* No data are available.

(7) *Diluent.* The basic diluent is physiologic saline/glycern with 0.5 percent phenol. These substances are widely used and are not known to have caused sensitization. Some producers add other substances (such albumin) to "stabilize" the active principle, to inhibit adsorption, or to act as a preservative (thimerosal). No evidence was found on the relationship of these substances, other than thimerosal, to sensitization.

(ii) *Test method*—(1) *Application.* Old Tuberculin is applied by the intracutaneous (Mantoux) or multiple puncture techniques. The Mantoux test is simple and accurate. The multiple puncture tests are simple but deliver a variable and undetermined quantity of test material into the skin.

(2) *Measurement.* The reaction to the test is a roughly circular area of induration, usually with clear-cut boundaries. The Mantoux test reaction is easily measured by trained observers. The multiple puncture methods sometimes result in one or several small reactions which may coalesce to form a circular reaction.

(3) *Quantitative dose response.* The Mantoux method produces a range of reactions that is sufficient for dose-response studies. These studies are more difficult with the multiple puncture methods because of the discontinuous distribution of reaction sizes resulting from coalescence of multiple small reactions.

(4) *Duration of reaction.* In a study of reactions caused by Tuberculin, PPD, it was found that mean induration size was relatively constant from 24 to 120 hours (Ref. 38). There is no reason to think that OT reactions would not have a similar pattern of duration.

(b) *Safety*—(i) *Host response.* Old Tuberculin has had widespread usage in millions of persons over many years, with great safety. There is no evidence that it produces anaphylaxis; carcinogenic, teratogenic or mutagenic effects; sensitization to the product; reactivation of tuberculosis; or interference with other medications. Infrequently, there may be a severe local skin reaction with vesiculation, pain and tissue necrosis. Very severe reactions may be associated with constitutional symptoms of fever and malaise as well as lymphangitis and lymphadenitis, but such reactions are rare. Localized scarring and change in skin pigmentation may occur infrequently following severe reactions.

(ii) *Test material*—(1) *Sterility.* The method of preparation can be expected to kill all microbial agents. The standard sterility tests seem to be sufficient for the particular substance.

(2) *Purity.* It is highly unlikely that any dead microbial agents are present. No tests are done for non-microbial contaminants. Varying amounts of the culture medium are present. The amount and nature of culture material will depend on the type of medium used to grow the tubercle bacilli. Other contaminants are highly unlikely.

(3) *Elimination by host.* No data are available.

(4) *Toxicity.* Old Tuberculin has been extensively used for approximately 80 years with no indication of toxicity when used intracutaneously in the usual dosages. Pinner and Valdrich produced progressive wasting disease in normal guinea pigs by repeated injection of culture filtrates (Ref. 39). The standard required safety tests should be sufficient to protect against the inadvertent introduction of toxic materials.

(iii) *Test method.* The intracutaneous Mantoux and multiple puncture methods of injection produce very little pain or bleeding, and are essentially noninjurious.

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TUBERCULIN, OLD (TINE TEST) BY LEDERLE

1. *Product.* Tuberculin, Old, (Tine Test) manufactured by Lederle Laboratories.

2. *Description.* The Lederle Tuberculin, Old, Tine Test is stated to be an intracutaneous test for the detection of tuberculin reactivity. It is recommended for screening rather than for diagnostic purposes (Ref. 1).

The Tine Test consists of a white plastic applicator with a stainless steel base, with four tines or prongs, each 2 mm in length. The tines are coated with concentrated, partially dried Old Tuberculin, and are protected by a removable plastic cap.

The Old Tuberculin (OT) is derived from culture filtrates of three strains of Mycobacterium tuberculosis (C, DT and PN) which were obtained in 1957 from Dr. J. M. Hejl, Chief, Biological Products Licensing Section, U.S. Department of Agriculture. The cultures are stored on solid egg medium at minus 60° C. At infrequent intervals, they are removed from storage, grown on veal tuberculin broth, replanted on solid egg medium, and stored again after adequate growth has occurred.

A production batch starts with a synthetic liquid medium containing asparagine, glycerine, dextrose and inorganic salts. Each bottle is inoculated with a piece of surface pellicle grown from one of the three seed cultures which have been incubated in veal infusion broth. After incubation, bottles which contain a surface pellicle and clear, unsedimented broth are heated, pooled, filtered, and concentrated. The concentrated filtrate is clarified and assayed for glycerin. Enough glycerin is then added to bring its concentration to 4.0 percent. Pooled concentrates representing the three seed cultures are autoclaved and tested for safety and sterility. If a batch is found to be contaminated, it is resterilized and tested again for safety and sterility. After all tests are satisfactory, the concentrates are pooled, and this mixture (bulk OT tested for potency, and again for safety and sterility.

The Tine Test unit marketed since 1965 is mechanically dipped into bulk OT. The concentrate is stabilized with acacia and lactose. The stabilized bulk OT is stored at minus 25° C until needed for dipping. Each Tine Test is dipped once by a mechanized automated process which deposits the stabilized OT on each tine. After dipping, a plastic cap is mechanically placed over the tines, and the entire unit is sterilized by ethylene oxide gas.

To apply the Tine Test, the tines are uncovered, and the unit is quickly and firmly applied to the cleansed skin of the forearm, holding the unit in place for approximately 1 second. Sufficient pressure should be applied to leave a circular depression on the subject's skin.

It is recommended that the test be read at 48 to 72 hours, and that any induration be measured. The current recommendations of the American Thoracic Society state that a vesicular reaction is tantamount to a positive Mantoux Test using 5 TU of PPD, and that any other reaction to the Tine Test resulting in induration measuring 2 mm or more in diameter should be rechecked with 5 TU of PPD by the Mantoux technique. The package insert submitted by the manu-

facturer, dated 12/73, still uses the old recommendations of the American Thoracic Society which were superseded in 1971 (Ref. 1).

3. *Analysis*—(a) *Effectiveness.* Most of the literature on the effectiveness of the Tine Test concerns the product marketed prior to 1966, which was hand-dipped into unstabilized OT. Because of changes in the manufacturing process, the following comments apply only to the current product unless otherwise stated.

Only one study was directly related to the sensitivity of the Tine Test (Ref. 2). Tine Tests were given to 84 patients hospitalized with bacteriologically proven pulmonary tuberculosis; all reacted with at least 2 mm of induration. Additional information submitted by the manufacturer on batch 104 showed that only 1 of 24 tuberculosis patients did not react to the Tine Test; this patient and one other did not react to 5 TU of PPD.

Other studies compare the Tine Test with a reference test using 5 TU of PPD given by the Mantoux technique. The degree of agreement of reactions produced by the Tine Test with those from the reference test are summarized in Table 1 (Refs. 3 through 6).

TABLE 1.—*Comparison of reactions from tine test with a reference test*

Reference PPD	Number of subjects	Copositivity	Conegativity	J[1]	Percent of false positive tine tests[2]
PPD–S	731	93.5	99.9	+93.4	2.3
PPD–S	589	98.4	85.9	+84.3	16.5
Not stated	645	100.0	74.7	+74.7	62.9
Do	26,236	92.1	96.5	+88.6	18.5

[1] J = Sensitivity plus specificity minus 100 pct (ref. 12).
[2] (Tine +, PPD−) + (total Tine +), in percent.

In only one study (Ref. 4) were the test sites randomized and the reactions read independently. For all studies, a positive Tine Test reaction was defined as 2 mm or more of induration. A positive reaction to PPD was defined as 6 mm or more induration in a study (Ref. 3) conducted among Eskimos in Alaska where heterologous reactions are almost unknown; in the other studies, a positive reaction to PPD was defined as 10 mm or more induration. The results indicate that the Tine Test identified nearly all positive reactors to 5 TU of PPD, i.e., the Tine Test has a high degree of co-positivity with the standard tuberculin test. It does less well in identifying negative reactors to PPD. The degree of agreement between the two tests, measured by the J statistic, is largely determined by the co-negativity. Among persons classified as positive Tine Test reactors, a considerable proportion were found to be negative to 5 TU of PPD by the Mantoux technique.

These indices of agreement varied markedly with geography in the large study conducted by the manufacturer (Ref. 6). The geographic areas were unfortunately not identified. A summary of the findings is given in Table 2.

TABLE 2.—*Comparison of tine test and Mantoux in 9 geographic areas*

Geographic area	Number of subjects	Copositivity	Conegativity	J[1]	Percent of false positive tine test reactions[2]
II	3,027	93.1	99.4	+92.5	10.8
III	2,621	85.5	[illegible]	+83.8	24.4
IV	6,754	92.1	95.1	+87.2	10.4
V	6,244	[illegible]	97.4	+84.0	54.4
VI	1,208	[illegible]	93.2	+86.1	20.5
VII	793	91.7	86.8	+78.5	27.4
VIII	585	71.1	99.6	+70.7	5.9
IX	7,299	95.3	95.1	+90.4	21.2
X	2,008	71.7	99.7	+71.4	13.1

[1] J=Sensitivity plus specificity minus 100 pct (ref. 12).
[2] (Tine +, PPD−) + (total Tine +), in percent.

In two areas, the Tine Test failed to identify almost 30 percent of the positive reactors to PPD. Co-negativity is good in all areas, but a high value of this index is expected whenever prevalence is low, as it was in this study. In one area, more than half of the Tine Test positives were negative to 5 TU of PPD given by the Mantoux technique. It should be noted that some of the variation noted between regions might have resulted from the use of different batches.

A fifth study is of limited value because reactions to PPD measuring 1 to 9 mm in diameter were not tabulated (Ref. 7). Among 111 patients admitted to medical wards who had 10 mm or more induration to 5 TU of PPD–S, 26 had Tine Test reactions of less than 2 mm, a co-positivity index of only 77 percent. A sixth study used tableted PPD which was then diluted and stored, raising the possibility of loss in potency from adsorption (Ref. 8). Using criteria for positive reactions of 2 mm or more for the Tine Test and 5 mm or more for the Mantoux, co-positivity was 99.0 percent and co-negativity 93.7 percent, giving a J value (defined in Table 1) of agreement of 92.7 percent. Only 7.8 percent of the Tine Test positives were not confirmed by the Mantoux technique.

Thus it appears that in most geographic areas, the Tine Test is capable of detecting practically all positive reactors to the standard tuberculin test, at a cost in some areas of producing a high proportion of positive reactions that are not confirmed by the Mantoux test with 5 TU of PPD. In some instances, the ability of the Tine Test to detect Mantoux reactors was less satisfactory. This seriously interfered with its value as a screening test. The reasons for the occasional poor co-positivity are not known but conceivably could have been due to failure to coat the tines in the dipping process or to differences in potency between tuberculin batches.

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material*—(1) *Composition.* The chemical composition of this particular product has not been studied. It should be noted that the manufacturer's protocol states that if a batch of tuberculin concentrate is found to be contaminated, it is resterilized and used. Such batches may contain products of bacteria other than *M. tuberculosis.*

(2) *Reproducibility.* The manufacturer submitted comparisons of reactions produced by Tine Tests made from 6 different batches with reactions produced by PPD given by the Mantoux technic (Ref. 6). Moderate variation was noted, but it is not possible to disentangle the effect of batch variation from the effects associated with geography.

3. *Standardization.* The tuberculin on the tines is standardized in a laboratory system utilizing guinea pigs sensitized by the injection of heat-killed tubercle bacilli suspended in mineral oil. At least four different dilutions of the material to be tested and similar dilutions of the U.S. reference standard are placed intracutaneously into specified sites on at least four guinea pigs. The materials tested for potency are the concentrated filtrate and the material eluted from the tines of the final product. The elution of the tines is done by placing 20 randomly selected Tine Tests units into a container with 10 ml of physiological saline and incubating them at room temperature for 1 hour with periodic shaking. For the concentrated filtrate, satisfactory potency is 3.2 to 4.8 times that of the U.S. Standard Old Tuberculine (an allowable variation of 20 percent); for the material eluted from the tines, the average potency for each Tine Test unit must be equivalent to 1/2700 to 1/1350 of the undiluted U.S. Standard Old Tuberculin (an allowable variation of 33 percent). A recent study has indicated that this method of standardization is inadequate (Ref. 11).

Standardization in humans has been done in the populations noted earlier (Refs. 3 through 8). Only one of these studies had a good experimental design, although the method of analysis may be criticized for omitting doubtful reactions in calculations of indices of agreement (Ref. 4). As can be seen in Tables 1 and 2, there was considerable variation in the results of the reported comparative studies. The variations may be related to the fact that most studies had poor experimental designs, to variations in the potencies of the Tine Test or reference tuberculins, or to true variation associated

with some unknown geographic factor. The evidence suggests that equivalence of the Tine Test with the standard Mantoux test among humans may be fairly claimed in some but not all studies.

Supplemental information from the manufacturer indicates that all batches have been compared with PPD by the Mantoux technic in humans including some tuberculosis patients (Ref. 13).

(4) *Stability.* Only two batches of tuberculin have been used long enough to yield meaningful information on stability. At 25° C, there appears to be little if any loss of potency insofar as this can be judged from the unrandomized tests done on only four guinea pigs and not read blindly.

(5) *Sensitization.* No data were given for this product. It is presumed that induction and augmentation of immunologic responses are generally similar to that for other tuberculin tests (*see* Generic Review on Old Tuberculin).

(6) *Elimination by host.* No data were given for this product. It is presumed that elimination is generally similar to that reported for PPD Tuberculin (*see* Generic Statement on PPD Tuberculin).

(7) *Diluent.* Documentation was provided by the manufacturer to demonstrate that the stabilizing and thickening agents cause no reaction and do not interfere with the reading of the tests (Ref. 17). In an interoffice report by D. H. Matson to R. N. Fallon dated May 27, 1966, it was documented that utilization of tuberculin-free tines carrying the stabilizing agents produced reactions that were no different from blank tines when tested in 100 human volunteers. Erythema of approximately 1 mm was common at 24 hours, but more severe reactions were almost never seen. The manufacturer also quotes a study by Kligman of Philadelphia which showed that acacia did not produce immediate or delayed reactions in the normal subject which would interfere with interpretation of a delayed skin reaction. MacLaren in California tested 10 atopic patients who had strongly positive intracutaneous tests to acacia in a dilution of 1:1000. These patients were tested with acacia-coated tines and naked tines as a control. All control reactions were negative but 8 of the 10 patients had immediate wheals around the penetration sites of the coated tines. At 24 hours, eight patients had no reactions, one had 10 mm of erythema, which was gone by the next day, and one did not return for a reading.

(ii) *Test method*—(1) *Application and dosage.* Application of the test is extremely simple. This is one of the major advantages of the test. The tines can be kept at hand in a desk drawer without refrigeration, as long as the temperature does not exceed 30° C. They can be used immediately without preparation except cleaning the skin. After use, the entire unit is discarded.

On the other hand, the dosage of tuberculin administered is not as accurate as a measured amount delivered by needle and syringe, as evidenced by the nonuniformity of the reactions around each of the four tines which is often noticed. Two studies have indicated that variation between duplicate Tine Test reactions is of similar magnitude to that resulting from duplicate Mantoux tests with 5 TU of PPD (Refs. 4 and 8). A smaller study on general hospital patients showed greater variation between duplicate Tine Test results (Ref. 7). In a fourth study, 100 positive tuberculin reactors were given three successive Tine Tests at 2-day-intervals (Ref. 9). The results of this study are summarized in Table 3.

TABLE 3.—*Results of 3 successive tine tests in positive tuberculin reactors*

Reaction criteria	Test 1	Test 2	Test 3
Percent less than 2 mm	0	31	14
Mean induration, millimeters	5.8	4.1	5.4

The cause of the reported variation between tests was not known, but variation in the amount of tuberculin delivered into the skin cannot be ruled out from the published data.

(2) *Measurement.* The area of induration is usually round, although partial coalescence of reactions around individual tines may produce an irregular area. None of the available reports indicate any difficulties in measurements.

(3) *Quantitative dose response.* The range of reaction sizes is more limited than for the Mantoux technic (Refs. 3, 5, and 8). Inability to control the dosage and the discontinuity in the distribution of reaction sizes resulting from coalescence of reactions around individual tines make dose-response studies difficult.

(4) *Duration of reaction.* The manufacturer recommends reading the reactions at 48 to 72 hours. In the data submitted, the agreement with the Mantoux technic was essentially the same for tests read at less than 96 hours and for those read at 96 hours to 144 hours (Ref. 6).

(b) *Safety*—(1) *Host response.* In six studies quoted here (Refs. 3 through 5, 7 through 9) no reference was made to any adverse reactions. In 31,051 Tine Tests made by 38 investigators for the manufacturer, only one severe inflammatory reaction was reported (Ref. 6). Many million units of Tine Tests have been sold over a 5-year period, and only 13 incidents of possible adverse effects were reported to the manufacturer. Nine of these reactions were allergic and varied from hives to angioneurotic edema. The remaining four were pain and bleeding, a dark nodule at the application site, a streptococcal cellulitis, and a severe local reaction.

As in any tuberculin test, severe skin reactions can occur occasionally with vesiculation and necrosis. In some of these, lymphangitis and lymphadenitis and symptoms of malaise and fever may occur for a day or so. Rarely, in severe reactions, scarring and changes in pigmentation may occur.

A single fatality, which occurred a few minutes after receiving a Tine Test has been reported (Ref. 14). No determination was made on the etiology of this occurrence (Refs. 15 and 16) Reactivation of disease and interference with other medications have never been reported.

The Panel found no information concerning potential carcinogenic, teratogenic, or mutagenic effects from this product.

Sensitization to tuberculin in this dosage is unlikely. The possibility of sensitization to acacia is unknown.

(ii) *Test material*—(1) *Sterility.* Tests for viable mycobacteria, both by guinea pig inoculation and by culture, are done on the pooled concentrated filtrates. Although the manufacturing process permits resterilization of bulk OT, the company has reported that no resterilization has been required for at least the last 15 years.

Sterility tests on the final product are performed on 40 Tine Test units from each sterilizer load of unspecified size. Half of the discs from the units are cultured in 20 tubes of thioglycollate medium, and half are cultured in 20 tubes of soybean-casein digest medium.

Nonbacterial agents are unlikely to be introduced into the manufacturing process. In addition, mycoplasma agents are not likely to be present nor to survive drying on the tines.

(2) *Purity.* Other contaminants are unlikely, except for the possibility of products from bacterial contaminants in batches that were resterilized because of the presence of such agents.

(3) *Elimination by the host.* There were no specific data on elimination of the material by the host.

(4) *Lack of toxicity.* Massive human experience suggests that toxicity does not occur. The material from 24 Tine Test units is eluted in 6 ml of saline. Two guinea pigs are injected with 5 ml of a 1:4 dilution of the eluate, and two mice are injected with 0.5 ml of this dilution. Over the past 5 years, an average of more than 60 batches per year have been so tested. Only two batches failed the initial safety test, probably as a result of animal colony epidemics.

(iii) *Test method.* The Tine Test causes slightly more pain than the Mantoux technic according to a trial among 41 students at the Johns Hopkins School of Hygiene (Ref. 10). However, pain is fleeting and slight. Bleeding is occasionally seen but is very slight. The test is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends that this product should be placed in Category I because the present product has been demonstrated to be safe and effective.

(b) Since there is ample evidence that batches of tuberculins may vary widely in potency, it is necessary that each new batch be tested for potency (sensitivity) in infected humans. Potency tests in guinea pigs do not necessarily reflect potency in human beings. Since the test is to be used for screening purposes, it is necessary to test only for sensitivity and not specificity. The Panel recommends

that sensitivity be sufficient to elicit positive reactions in 100 percent of at least 50 persons who are known to have had bacteriologically confirmed tuberculosis and who are tuberculin positive as demonstrated by a simultaneous Mantoux test with 5 TU of PPD. Other conditions are recommended for continued marketing.

(c) Quality control: Because of the possibility that the mechanical dipping process may occasionally fail to deposit tuberculin on the tines of an individual unit, routine testing of the final product should include a test for protein or other test materials on the tines of randomly selected individual units.

(d) Labeling changes: (i) If the American Thoracic Society recommendations are to be included in the labeling, the current version should be used.

(ii) Change the precautions to state that the material used for the confirmatory Mantoux (intracutaneous) test shall be 5 TU Tuberculin, PPD.

(iii) Delete references implying equivalent significance of reactions to this product and to those of 5 TU of Tuberculin, PPD by the Mantoux test.

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TUBERCULIN, OLD (MANTOUX TEST) BY ELI LILLY

1. *Product.* Tuberculin, Old, Human, for Mantoux Test, U.S.P. manufactured by Eli Lilly and Co.

2. *Description.* Tuberculin, Old, Human, for Mantoux Tests, U.S.P. as prepared by Eli Lilly and Company is distributed in a package containing two vials. One vial contains Old Tuberculin (OT) representing approximately 0.1 ml of concentrated OT diluted 1:100 with 50 percent glycerin and preserved with 0.5 percent phenol. The other vial contains sodium chloride injection with 0.5 percent phenol in sufficient volume that its injection into the vial containing OT will produce a test solution of 1:1000 OT. This final solution is said to contain 0.1 mg OT per 0.1 ml, or 10 U.S. units (TU) per 0.1 ml.

This OT is prepared from human tubercle bacilli, strain H–37, received on 4 September 1932 from the U.S. Public Health Service. The organism was maintained on "TB agar slants" from 1951 to the present. The last batch of tuberculin concentrate was produced in May, 1968 after the organism had been grown on glycerin-veal infusion-peptone broth for at least 6 weeks. Flasks that appeared to be uncontaminated were heated in flowing steam for 1½ hours. The flask contents were then filtered through sterile filter paper and diatomaceous earth. The filtrate was evaporated to one-tenth of its original volume, preserved with phenol at a final concentration of 0.5 percent, and stored in the chill room at 2° to 5° C for at least 1 month. It was then diluted 1:100 with 50 percent glycerin containing 0.5 percent phenol, and stored again in the chill room until measured into vials for distribution.

3. *Analysis*—(a) *Effectiveness.* No evidence was presented to show that OT from the current batch has ever been tested for sensitivity or specificity in humans, or that it has ever been compared to a standard tuberculin in humans.

Each lot is standardized in four sensitized guinea pigs without randomization of test sites or "blind" readings. The procedure used by Lilly (and others) has been shown to be inadequate for standardization (Ref. 1). This OT has not been tested in animals for specificity, nor for co-negativity with a standard tuberculin.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material.*—(1) *Chemical composition.* It has not been chemically defined.

(2) *Reproducibility.* Batch-to-batch variability seems to be a minor problem since the batch in current use was prepared in 1968. No data were given on reproducibility from lot-to-lot except that each lot passes the usual potency test using four guinea pigs, nonrandom test sites, and nonblind readings. Lots are produced at approximately yearly intervals.

(3) *Standardization in laboratory system.* Old Tuberculin can be standardized in guinea pigs against a standard tuberculin preparation if a sufficient number of animals are used and if the proper experimental design is followed (Ref. 1). There is evidence that the results in guinea pigs may not be directly transferable to man (Refs. 2 and 3), although there is no information on this point for this particular product.

(4) *Stability.* The only data furnished on stability referred to batches prepared prior to the date of the present batch. Not only are the findings of little relevance, but the experimental design was improper because it lacked randomization of test sites and blind readings. In addition, the fact that the majority of reactions were measured as 5 or 10 mm suggests that little care was taken in measurement.

(5) *Sensitization.* Sensitization studies were conducted in nine guinea pigs using a batch of OT prepared prior to the one in current use. The findings were interpreted as showing that the amount of phenol in the large challenging dose was at the toxic level for guinea pigs. No data were available on the currently used batch.

(6) *Elimination by the host.* No data were given.

(7) *Diluent.* The diluent is generally considered not to cause sensitization or reactions. In 1964, the diluent was reported to have caused no reactions in any of 268 male prisoners.

(ii) *Test method.*—(1) *Application of test.* The Mantoux method of intracutaneous injection is relatively simple and accurate.

(2) *Measurement of reactions.* Although there was no information on this particular product, there is no reason to believe that the reactions differ qualitatively from those produced by other Old Tuberculins. The reactions are presumably round with clearcut boundaries.

(3) *Quantitative dose response.* There were no data on this particular product except for the standardization results of each lot in guinea pigs. It appears that it can produce a range of reaction sizes that is adequate for dose-response studies.

(4) *Duration of reaction.* No data were given. The package brochure states that reactions appear within 6 to 8 hours, reach a maximum size in 24 to 48 hours, and usually disappear in 6 to 10 days.

(b) *Safety.*—(i) *Host response.* No specific data were available on adverse reactions to this product. The package brochure states that severe local reactions uncommonly occur at the injection sites and that in such instances necrosis and scar formation may occur. Presum-

ably, constitutional symptoms may accompany some severe local reactions.

It is possible that some of the broth components, particularly the veal infusion, may be sensitizing.

The Panel found no information on potential carcinogenic, teratogenic, or mutagenic effects from this product.

(ii) *Test material.*—(1) *Sterility.* As a check on the absence of viable tubercle bacilli, an unspecified number of guinea pigs is injected with an unspecified amount of each lot of concentrate, observed for 6 weeks, and necropsied to check for tuberculous lesions.

For a bulk sterility check, two vessels of fluid thioglycollate medium are inoculated with 5 ml of test material and incubated for 14 days. For a filled sterility check, 20 smaller vessels of fluid thioglycollate medium and 20 vessels of trypticase soy broth are inoculated "with the entire contents of the vials of test vaccine (sic) per vessel" and incubated for 14 days. If growth is found in any of the filled sterility tests, two retests may be performed, the second retest using twice the number of vessels and being allowed only if the bulk sterility check is negative.

No tests are done for viruses, mycoplasma, or fungi.

(2) *Purity.* No tests are done for contaminants.

(3) *Elimination by host.* No data were given.

(4) *Lack of toxicity.* The manufacturer stated that only two complaints have been received in the past 5 years. In one instance, a physician complained that reactions were not produced in known tuberculin positive patients; and in the other, strongly positive reactions (15–20 mm) were noted in six healthy infants.

Two guinea pigs are injected subcutaneously (sic) with 5 ml of test material at 1:1000 dilution and 2 mice are injected intraperitoneally with 0.5 ml of the same dilution. All animals must survive for 7 days without obvious symptoms.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends that this product should be placed in Category IIIB, license to be suspended, because there is inadequate evidence of standardization, no evidence of effectiveness in humans, and a question of safety. Before the license is reinstated, acceptable data based on scientifically sound methods should be submitted to demonstrate effectiveness in humans (Refs. 4 and 5). These data should include for each batch:

(i) Comparison of the product with the Tuberculin standard (PPD) by means of dose response curves in humans and animals.

(ii) Distribution of reactions in persons known to be infected and in those presumed to be uninfected with *M. tuberculosis* and other mycobacteria.

(iii) Chemical characterization (including protein, carbohydrate, lipid and nucleic acid).

(b) In addition the manufacturer should:

(i) Provide evidence of stability of the material in the final container (i.e., possible adsorption to glass or plastic).

(ii) Demonstrate that:

(1) The veal antigens in the product do not sensitize.

(2) The veal antigens in the product do not elicit false positive reactions in sensitized individuals.

(c) Labeling: The product brochure dated October 1974 should include the latest American Thoracic Society (ATS) recommendations for the interpretation of the skin test. Departures from the ATS recommendations should be justified.

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(1) Wijsmuller, G., A. Bardine, P. Rust, et al., "Evaluation of the Animal Assay of Tuberculin Intended for Use in Humans. I. Reproducibility," *Journal of Biological Standardization*, 3:51–63, 1975.

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OLD TUBERCULIN BY MASSACHUSETTS PUBLIC HEALTH BIOLOGIC LABORATORIES

1. *Product.* Old Tuberculin manufactured by Massachusetts Public Health Biologic Laboratories (MPHBL).

2. *Description.* Massachusetts Public Health Biologic Laboratories Old Tuberculin (OT) is prepared as a concentrated sterile culture filtrate of strain H–37 tubercle bacilli originally obtained in 1929 from Saranac Laboratory. Organisms are grown in gelatin hydrolysate medium containing 5 percent glycerin. The concentrated bulk product is preserved with 0.5 percent phenol. The diluted preparation contains 0.4 percent human albumin as a stabilizer (Ref. 1).

The stock concentrate was prepared in 1947 by incubation of 10-day seed cultures at 35° C for 8½ weeks. This stock culture was heat sterilized (1 hour at 100° C), followed by cheese cloth filtration and concentration of the filtrate to 1/10 of the original volume by vacuum evaporation. It was then dialyzed against 0.5 percent phenol solution and Seitz-filtered. This material was designated as Lot OT–48. Several sublots were prepared in phosphate buffer containing 50 percent glycerin, one of which was the current lot OT–48–B–3B. Current material is prepared approximately every 6 months by diluting a portion of the sublot (OT–48–B–3B) to a concentration said to be sufficient to make each 0.1 ml of this diluted material equivalent to 5 TU of the U.S. OT standard. The diluted product is dispensed in 2 ml vials.

3. *Analysis.*—(a) *Effectiveness.* In 1972 data were obtained on comparison of this product's potency with that of standard PPD-tuberculin stabilized with Tween-80 (the latter having been standardized against PPD–S, the International Reference Standard). In this study (Ref. 2), 191 employees of a tuberculosis treatment facility were tested and the following results were obtained:

MPHBL-OT	PPD-tuberculin with tween		
	Positive[1]	Negative	Total
Positive	65	0	65
Negative	15	111	126
Total	80	111	191

[1] Positive, equal to or greater than 10 mm induration.

No individuals who were negative to PPD were positive to MPHBL–OT. Sixty-five employees positive to PPD were also positive to MPHBL–OT, but 15 positive to PPD were negative to MPHBL–OT. Results of this study indicate that MPHBL–OT was less potent than PPD but not necessarily less specific. No pertinent data are given with regard to specificity of MPHBL–OT. In an earlier study (1960), a comparison was made of mental hospital patients between PPD–S (not stabilized with Tween) and MPHBL–OT (Ref. 3). Based on the absolute numbers of reactors (rather than percentages) the results of this study are summarized as follows:

MPHBL-OT	PPD-S		
	Positive	Negative	Total
Positive	917	8	925
Negative	316	673	989

In this study, 917 (74 percent) of 1233 of the individuals were positive to both PPD–S and MPHBL–OT, but 316 were positive to PPD–S and negative to MPHBL–OT. Results indicated considerably less potency for the MPHBL product when compared with PPD–S. The producer attributed the lesser potency at that time to the use of gum arabic as a stabilizer (Ref. 4). At a later date, human albumin was substituted for gum arabic (although animal data suggested that Tween-80 was equal to albumin in stabilizing potency).

(i) *Test material*—(1) *Chemical composition.* The final diluted crude culture filtrate mixture contains OT concentrate (tubercle bacillus products and glycerin broth components), human serum albumin, boric acid, sodium borate, sodium chloride and thimerosal. The chemical composition of OT concentrate is not further defined.

(2) *Reproducibility.* No data were available.

(3) *Standardization.* Efficacy data provided by the manufacturer state that tests are carried out as specified in the

Code of Federal Regulations, Title 21, Subchapter F, § 610.11 (general safety tests) plus §§ 650.11 and 650.14. (The usual potency tests compare the product in at least 4 guinea pigs against the U.S. Standard OT.) No data were provided however, other than the two studies previously referred to under "Effectiveness." It should be noted that these producers are among those who have called attention to the fact that different results can be obtained in guinea pigs and in man.

(4) *Stability.* In an internal memorandum dated March 1962, the manufacturer reported that an MPHBL–OT product, with human albumin as the stabilizer, after 7 months storage at 5° C actually resulted in slightly larger delayed skin reactions in 23 human volunteers than did freshly diluted material. The details of the skin testing procedure and reading of reactions were not provided. If these tests were properly performed and measured, diluted MPHBL–OT stabilized in normal human serum albumin is stable over this time interval. In other potency studies in man using MPHBL–OT with human albumin as stabilizer, a 16 percent decrease in delayed skin reaction size was noted in OT stored at 35° C for 42 days when compared to OT stored at 5° C for the same period. Thus storage at approximate body temperature did not result in significant loss of potency over this time interval.

(5) *Sensitization.* No data were provided.

(6) *Elimination by host.* No data were provided.

(7) *Diluent.* The diluent contains thimerosal which has been reported to induce hypersensitivity. The Panel's comments regarding the sensitizing capacity of thimerosal apply to this product. See the statement on "Thimerosal as a Preservative." The package insert, however, implies that the only preservative is phenol. This discrepancy has been clarified in a recent letter from Dr. Grady, the Laboratory Director who stated: "Our circular apparently bears an historical error in stating the preservative to be 0.5 percent phenol. This stems from the time when we distributed only the concentrates and the consumer was obliged to prepare his own dilutions. These concentrates then and now are indeed preserved with 0.5 percent phenol. They are diluted 1:1000 to 1:2000 to prepare the diluted clinical product which renders the phenol content negligible and thimerosal, 1:30,000 is provided in the diluent." The diluent also contains human serum albumin which is not known to be immunogenic in man (except in very rare individuals with analbuminemia).

(i) *Test method*—(1) *Application.* The product is inoculated intracutaneously by the standard Mantoux method in 0.1 ml volume. This method is reasonably simple and accurate.

(2) *Measurement.* The reactions produced are similar to those induced by tuberculins.

(3) *Dose response.* No data were provided.

(4) *Duration of reaction.* The duration of reaction is presumably similar to that observed for other tuberculin preparations.

(b) *Safety*—(1) *Host response.* Only anecdotal information was given, namely that millions of doses have been dispensed without reported adverse reactions and that MPHBL–OT is comparable to PPD solution without Tween-80 in its lack of production of adverse reactions (Ref. 5). The Panel found no information on potential carcinogenic, teratogenic, or mutagenic effects from this product. As in the case of other delayed hypersensitivity skin test products, there is no reason to believe that testing with MPHBL–OT causes excessive pain, skin irritation, or scarring.

(i) *Test material.*—(1) *Sterility.* No data were provided except for the statement that the required sterility tests are performed.

(2) *Purity.* No data were provided on product purity. The generic statement on purity presumably applies to MPHBL–OT.

(3) *Elimination by host.* No data were available.

(4) *Toxicity.* No untoward toxic reactions have been reported (the manufacturers have dispensed several million doses during the past 5 years).

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends that this product should be placed in Category IIIB, license to be suspended, because the available evidence supplied by the manufacturer demonstrates that the product failed to identify a significant portion (19 percent) of the tuberculin positive population when compared to the PPD standard. Before the license is reinstated, acceptable data based on scientifically sound methods should be submitted to demonstrate effectiveness in humans (Refs. 6 and 7). The data should include for each batch:

(i) Adequate steps to ensure that the product has the proper potency.

(ii) Comparison of the product with the tuberculin standard (PPD) by means of dose response curves in humans and animals.

(iii) Distribution of reactions in persons known to be infected and in those presumed to be uninfected with *M. tuberculosis* and other mycobacteria.

(iv) Chemical characterization (including protein, carbohydrate, lipids and nucleic acids).

In addition the manufacturer should provide evidence of stability of the material in the final container (i.e., possible absorption to glass or plastic).

(b) Labeling: The following information, in addition to that in the product brochure dated August 1968, should be included in the labeling:

(i) State that a positive skin test may indicate infections with other mycobacteria.

(ii) Delete paragraph one under "Discussion" since this is erroneous.

(iii) While pertinent references are highly desirable, references 1, 2, and 3 should be deleted as unrelated to the product.

(iv) A precaution against use in thimerosal sensitive subjects.

(v) The latest American Thoracic Society (ATS) recommendation for the interpreation of the skin test should be stated. Departures from the ATS recommendations should be justified.

REFERENCES

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(2) Tose, L., S. C. Cohen, and D. S. Sherman, Correspondence: "PPD with Tween and Massachusetts OT Compared," New England Journal of Medicine, 286:1419, 1972.

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(7) "Guidelines for the Comparative Testing in Humans of New Tuberculin Batches and New Methods of Application," Proceedings of the Second National Conference on Evaluation of Procedures for Tuberculin Testing, Center for Disease Control, U.S. Public Health Service, Atlanta, Ga. January 8–9, 1970.

OLD TUBERCULIN BY TEXAS STATE DEPARTMENT OF HEALTH

1. *Product.* Old Tuberculin manufactured by Texas State Department of Health.

2. *Description.* Texas State Department of Health Old Tuberculin is a sterile concentrated culture filtrate from tubercle bacilli grown on Dorset's medium. Seed cultures for this product are Dorset strains PN, DT, and C obtained in 1945. After the organisms have been killed, the filtrate is concentrated and adjusted to a final concentration of 10 or 100 U.S. units of tuberculin per 0.1 ml. The diluent is a solution of borax, boric acids and sodium chloride, containing 0.04 percent gum arabic, and a final concentration of 0.25 percent phenol as a preservative.

3. *Analysis.*—(a) *Effectiveness.* No information was submitted to show that the material has ever been tested in humans for sensitivity or specificity. Other than the standardization procedure, the only test remotely resembling a test for sensitivity and specificity is the intracutaneous injection of final container material into each of the two guinea pigs, one sensitized and the other not sensitized.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material.*—(1) *Chemical composition.* No chemical definition of the product was submitted except what

could be inferred from the method of preparation.

(2) *Reproducibility.* No data were provided on lot-to-lot reproducibility except by a communication on March 28, 1975, from Mr. Philip J. Forsyth, Responsible Head of the Biologics Production Division of the Texas State Department of Health, in which he said that "Our last twelve lots (M–21 through M–26, and N–21 through N–26) were made up from the same concentrate using the same proportionate dilutions with no observable change in potency over the past 15 years • • •." The above lots in diluted form were compared with the U.S. standard and in every instance met the currently required criteria for equivalency, which have been shown to be capable of detecting only gross variations in potency (Ref. 1).

(3) *Standardization.* This is carried out only by the required procedure using four sensitized guinea pigs, without randomization of test sites and without "blind" readings. No information was submitted to indicate whether or not this procedure is comparable to standardization in human subjects.

(4) *Stability.* The only specific data were provided by accelerated stability tests at 37° C for 20 days. The manufacturer points out that this is highly stable material because lots in current use were made from concentrated stock made up and tested in 1959. The manufacturer states that lots in final diluted form have been successfully retested several times for extension of dating periods and have maintained potency.

"• • • Current lot N–26 was first tested in April 1969, and then retested in March 1972, April 1972, and March 1974." While no specific stability tests are available beyond 20 days at 37° C, there is no reason to suggest that this product is less stable than any other Old Tuberculin.

(5) *Sensitization.* No data were given.

(6) *Elimination by host.* No data were given.

(7) *Diluent.* The final lot is made up by diluting the stock concentrates using a borax-boric acid sodium chloride solution containing 0.04 percent acacia and a final concentration of 0.25 percent phenol.

(ii) *Test method.*—(1) *Application.* The material is administered by the Mantoux method, which is reasonably accurate and simple.

(2) *Measurement.* Although no data were available, there is no reason to doubt that this material produces round reactions with reasonably clear-cut borders similar to those produced by other tuberculins.

(3) *Quantitative dose response.* No data relative to this were submitted, but there is no reason to believe that this material cannot be studied in this fashion, as is the case for other tuberculins.

(4) *Duration of reaction.* There is no reason to believe that reactions produced by this product do not follow a time sequence similar to those produced by other tuberculins.

(b) *Safety.*—(i) *Host response.* No specific information was submitted. However, there is no reason to believe that this product differs from other tuberculins in this respect.

(ii) *Test material.*—(1) *Sterility.* The required tests are performed.

(2) *Purity.* No information was submitted, but the statement in the generic review probably applies to this product also.

(3) *Elimination by host.* No data were available.

(4) *Toxicity.* The standard safety tests are performed. In the past 10 years, over a million doses have been distributed without adverse reactions having been reported to the producer. The producer noted, when queried, that they have no evidence that acacia in their product has caused sensitization, although acacia is known to have this capability.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends that this product should be placed in Category IIIB, license to be suspended, because there are no data on standardization, sensitivity, specificity, and effectiveness in humans. Before the license is reinstated acceptable data based on scientifically sound methods should be submitted to demonstrate effectiveness in humans (Refs. 1 and 2). These data should include for each batch:

(i) Comparison of the product with the Tuberculin standard (PPD) by means of dose response curves in humans and animals.

(ii) Distribution of reactions in persons known to be infected and in those presumed to be uninfected with *M. tuberculosis* and other mycobacteria.

(iii) Chemical characterization (including protein, carbohydrate, lipid and nucleic acid).

In addition the manufacturer should provide evidence of stability of the material in the final container, i.e., possible adsorption to glass or plastic.

(b) Labeling: The following revisions should be made in the labeling:

(i) The manufacturer should delete any reference to treatment.

(ii) The manufacturer should use the latest American Thoracic Society recommendations for the interpretation of the skin test unles departures from these recommendations are justified.

(iii) The manufacturer should include a warning that the product should not be used in acacia (gum arabic) sensitive individuals.

REFERENCES

(1) Wijsmuller, G. and A. L. Bardine, "A Method of Characterizing Tuberculins," *American Review of Respiratory Diseases*, 105:736–746, 1972.

(2) "Guidelines for the Comparative Testing in Humans of New Tuberculin Batches and New Methods of Application," *Proceedings of Second Conference on Evaluation of Procedures for Tuberculin Testing*, Center for Disease Control, U.S. Public Health Service, Atlanta, Georgia, January 8–9, 1970.

TUBERCULIN, OLD BY INSTITUT MERIEUX

1. *Product.* Tuberculin, Old manufactured by Institut Merieux, Lyons, France, Distributed by Lincoln Laboratories, Inc., Decatur, Illinois, Brand Name "Mono-Vacc."

2. *Description.* Institut Merieux, Tuberculin, Old (Mono-Vacc) is used mainly as a screening test to evaluate delayed-type immunity to *Mycobacterium tuberculosis and M. bovis* infection (Ref. 1).

The Old Tuberculin (OT) for Mono-Vacc Test produced by Institut Merieux is packaged in sterile, plastic, disposable, multiple puncture devices. Each test dose is stated to be comparable to or greater than 5 tuberculin units (TU) administered by the Mantoux method. For the production of OT, three human isolates of *M. tuberculosis* (C, D, and PN) and one bovine isolate (Vallee) are cultured separately on a synthetic medium. At the end of the incubation the cultures are autoclaved and the filtrates harvested. Equal parts of the filtrates are pooled, glycerin is added and the pooled filtrates are concentrated. After control tests are performed, one or several lots of OT are mixed with glycerin to adjust the titer of the final antigen to 300,000 units/ml. This material, after sterility and safety testing, is placed in small plastic tubes which cover the spikes on applicators designed to fit over the thumbs of the testers.

3. *Analysis.*—(a) *Effectiveness.* The effectiveness of the Mono-Vacc OT skin test to measure the presence of immunologic experience with *M. tuberculosis* or *M. bovis* has been evaluated by many workers (Refs. 2 through 6, 8, 9, and 11). None of these studies was conducted in a randomized, blind fashion.

Two studies have compared the Mono-Vacc with PPD in bacteriologically confirmed cases of tuberculosis (Refs. 8 and 11). The PPD in the first study was a well-standardized product, 2 TU of RT–23 with Tween, which differs somewhat in its characteristics from PPD–S (Ref. 10). Of 69 patients, 68 had 2 or more mm of induration to the Mono-Vac; 67 of these 68 also had reactions of 5 mm or more to RT–23. The second study found that all of 84 bacteriologically positive tuberculosis patients had at least 2 mm of induration to the Mono-Vac (Ref. 11). These results indicate a high degree of sensitivity of the Mono-Vacc (98.6 percent).

The remainder of the studies gave values based on comparisons with another tuberculin test, and yielded figures for co-positivity and co-negativity rather than for sensitivity and specificity. Several studies submitted by the manufacturer did not compare the Mono-Vac with PPD or RT and hence will be ignored. Among the remainder, only two used a PPD known to be well-standardized. The others either used an unspecified PPD or the old Parke-Davis tableted PPD. A summary of co-positivity and co-negativity from the literature (Refs. 2, 3, 4, 5, 8, 9, and 11) are given below:

Number of subjects		Percent Copositivity	Percent Conegativity
Positive[1]	Negative[1]		
279	1,127	[illegible]	96.5
87	1,872	100.0	96.2
256	1,167	98.8	91.6
60	1,962	100.0	99.0
105	248	96.4	85.1
258	412	98.6	81.9
80	[2] 4	100.0	0

[1] Results of test with PPD; positive is 10 or more millimeters induration.
[2] These 4 tuberculosis patients had PPD reactions of 5 to 9 mm, and would be classified as positive by the American Thoracic Society standards.

The above studies were not done in areas with high frequencies of nontuberculous mycobacterial infections and hence undoubtedly overstate the conegativity of the Mono-Vacc. Moreover, there is ample evidence in the literature that batches of tuberculins may vary widely in potency. Following a direct request, the manufacturer did provide evidence of effectiveness in humans of the presently marketed product (Ref. 14). The manufacturer considers a positive reaction as the appearance of an indurated reaction of 1 mm or more.

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material*—(1) *Chemical composition.* The concentrated OT dispensed in the Mono-Vacc Test is not chemically defined. Assays of protein nitrogen were performed but the results were not reported to the panel.

(2) *Reproducibility from lot-to-lot.* Each lot of concentrated OT prepared for the Mono-Vacc Test is standardized by intracutaneous tests in hypersensitive guinea pigs, comparing the reactions with those produced by the standard OT. The potency of each lot is adjusted so that it has a titer of 300,000 units/ml plus or minus 20 percent. Dose response curves for batches k0008/1 and k0398/1 differ but not significantly.

(3) *Standardization.* The standardization of each lot is accomplished by injecting 0.5, 1, 2, and 4 TU of the U.S. Standard tuberculin (Bureau of Biologics) intracutaneously by the Mantoux method into hypersensitive guinea pigs and comparing the degree of reactivity to four comparable levels of activity of the test lot.

It has been shown recently that the number of animals must be larger than the four required by regulations if the required accuracy is to be achieved (Ref. 7). Also there is evidence that the results in guinea pigs may not be directly transferable to man (Refs. 12 and 13) although there is no information on this point for this particular product.

(4) *Stability.* A comparison was made between recently manufactured Mono-Vacc Tests and Mono-Vacc Tests that were 15 months old in 34 subjects. There is no indication that the test sites were randominzed or the readings made blindly. Thirty-two of the 34 comparisons gave identical readings for each of the 2 tests, while the other 2 comparisons deviated by only 1 mm. Such close agreements are rarely, if ever, achieved when readings are done blindly. No further tests of the stability of the finished product, i.e. OT on the Mono-Vacc spikes, were reported to the panel.

Four lots of OT tested against the U.S. Standard OT after 2 to 3 years of storage had potencies, according to the usual four-guinea-pig test, equal to or greater than the standard.

(5) *Sensitization.* Sensitization studies were performed on normal guinea pigs weighing about 400 g. Each animal received three successive 0.1 ml intracutaneous injections, at 7-day intervals, of OT diluted to 1/60 (5,000 units). Animals were challenged at 2 to 4 weeks with the same dose of OT, at which time their reactions were no larger than those produced in animals receiving their first injection.

(6) *Elimination by host.* No data were presented regarding elimination of the concentrated OT from the host.

(7) *Diluent.* Glycerin is employed as diluent for the concentrated OT in order to achieve a final potency titer of 300,000 units/ml plus or minus 20 percent. The sample must not contain more than 20 mg of glycerin in the 10 ml sample.

(ii) *Test method*—(1) *Application of test.* (a) The test requires pressing the Mono-Vacc spikes, on which a "drop" of concentrated OT is deposited, into the cleansed volar surface of the arm. The instrument is a plastic ring with a 9 mm square setting on which is mounted a 3 mm "stone" possessing nine spikes (2 mm long) arranged in three rows of three each at a distance of 0.75 mm from each other. The test procedure is extremely simple.

(b) The dosage injected is not controlled. The test relies on the ability of the nine spikes on the ringed instrument to penetrate the skin and deposit an unknown quantity of concentrated OT (300,000 units/ml).

(2) *Measurement of test.* (a) The positive reaction appears to be round.

(b) The producers states that "• • • light and local erythema is observed but the sole criterion for a positive reaction is palpable induration. The presence of palpable induration (even 1 to 2 mm) indicates a positive reaction."

(3) *Quantitative dose response.* The test is available in only one concentration of OT, 300,000 units/ml. There were no data submitted in reference to dose response as directly applicable to the Mono- Vacc Test.

(4) *Duration of reaction.* The instructions state that the reaction be read at 72 to 96 hours after administration of the test. A review of the literature reveals that many reactions from the Mono-Vacc Test have been read as early as 48 hours (Refs. 3, 4, and 6).

(b) *Safety.*—(1) *Host response.* The Panel found no information on potential carcinogenic, teratogenic, or mutagenic effects from this product.

In highly senstive subjects, severe local reactions can occur, with localized vesiculation and necrosis, occasionally accompanied by lymphangitis and lymphadenitis and mild constitutional reactions such as fever and malaise. In such instances, scarring and change in pigmentation are possible. Pain on administration is minimal.

There is no reason to believe that this product differs significantly from other tests using OT.

(ii) *Test material*—(1) *Sterility.* Each bulk lot of concentrated OT is tested for sterility beyond the requirements of the Bureau of Biologics.

(2) *Purity.* No tests are performed for purity, except as noted under "Toxicity."

(3) *Elimination by host.* No data were available.

(4) *Toxicity.* Experience with the test in humans suggests that the concentrated OT is nontoxic as used in the test. In the 5-year-period 1969–1973, many million Mono-Vacc Tests were distributed. The U.S. distributor has had no reports of adverse reactions. The plastic material for the Mono-Vacc applicator is checked for excess acidity and alkalinity, arsenic, tin, and heavy metals. Physiological saline which has been autoclaved with the plastic is also injected into mice to check for soluble toxic materials. The general safety tests are more extensive than required by Bureau of Biologics regulations.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends this product should be placed in Category I because the present product has been demonstrated to be safe and effective.

(b) Since there is ample evidence in the literature that batches of tuberculin may vary widely in potency, it is necessary that each new batch be tested for potency (sensitivity) in infected humans. Potency tests in guinea pigs do not necessarily reflect potency in human beings. Since the test is to be used for screening purposes, then it is necessary to test only for sensitivity and not specificity. The Panel recommends sensitivity sufficient to elicit positive reactions in 100 percent of at least 50 persons who are known to have had bacteriologically confirmed tuberculosis and who are tuberculin positive as demonstrated by a simultaneous Mantoux test with 5 TU of PPD.

(c) Labeling changes should indicate that since this test is a screening procedure, positive Mono-Vacc reactions should be confirmed by a 5 TU Tuberculin, PPD Mantoux (intracutaneous) and possibly other diagnostic test procedures. It is not correct to imply that the test does not cause any pain or bleeding.

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TUBERCULIN, PPD

GENERIC STATEMENT

Purified Protein Derivative of tuberbulin (PPD) is a diagnostic skin test reagent prepared from a protein precipitate of filtrates of sterilized cultures of tubercle bacilli. It is used primarily to detect delayed hypersensitivity in individuals who have had immunologic experience with *Mycobacterium tuberculosis* and *Mycobacterium bovis*.

Tuberculin, PPD was originally produced by Seibert in 1934. Her lot 49608, produced in 1939, is the International and the United States Reference Standard and is designated PPD–S. The international and United States tuberculin unit is the activity contained in a specified weight of PPD–S (1 TU=0.028 microgram (of which 0.008 microgram is sodium chloride)). Commercial PPD products labeled 5 TU (the standard test dose) are standardized against PPD–S to achieve potency which is biologically equivalent to 5 TU of PPD–S dissolved in Seibert's phosphate buffer. Products labeled 1 TU and 250 TU are calculated dilutions based on the dosage determined to be 5 TU, not on clinical evaluation (biologic equivalency in humans).

A positive tuberculin test usually develops 2 to 10 weeks after initial infection with *M. tuberculosis* or *M. bovis*. PPD possesses excellent, although not perfect, ability to identify all individuals with this specific immunologic experience. Although sensitivity tends to persist throughout life, it may wane or disappear in a variety of circumstances. When the usual causes for negative reactions are absent, failure to react to a tuberculin test is helpful in excluding the diagnosis of tuberculosis. Skin reactions to PPD may occur in individuals infected with mycobacteria other than *M. tuberculosis* or *M. bovis*.

Practically all of the valid data on sensitivity and specificity of PPD in the United States have been obtained from studies using the 5 TU dose. Increased sensitivity can be obtained by using higher dosage, but apparently only at the cost of decreased specificity.

Extensive investigation by increasingly sophisticated chemical and immunologic techniques has resulted in a wealth of information on the chemical composition of PPD. It is agreed that the major active principles are protein in nature. Potency can vary from batch to batch. Following batch standardization in human populations, it is possible to standardize PPD lots against PPD–S using an adequate number of sensitized guinea pigs.

Purified Protein Derivative is applied by the intracutaneous (Mantoux) or multiple puncture technique. The intracutaneous test is simple and accurate. The multiple puncture tests are simple but deliver a variable and undetermined quantity of test material in to the skin.

Purification Protein Derivative is stable for long periods under proper conditions of storage and has had widespread usage in millions of persons over many years, with great safety.

PRODUCT REVIEW

Purified Protein Derivative of tuberculin (PPD) is a protein precipitate obtained from filtrates of sterilized cultures of tubercle bacilli. It is used primarily as a diagnostic skin test reagent to detect delayed hypersensitivity in individuals who have had immunologic experience with *Mycobacterium tuberculosis* or *Mycobacterium bovis*.

Florence Seibert coined the term "purified protein derivative" in 1934. She made this product from heat concentrated synthetic medium (HCSM) Old Tuberculin by precipitating the protein with trichloroacetic acid. She later changed to precipitation with saturated ammonium sulfate to obtain a preparation with less nucleic acid and polysaccharide (Ref. 1). A large batch of PPD was produced by Seibert in 1939 to be used as a standard. This material was lot 49608 which was designated PPD–S (Ref. 2). It was adopted by the World Health Organization in 1951 as the International Standard for Purified Protein Derivative of Mammalian Tuberculin. It is the United States reference standard. The International Unit for PPD of Mammalian Tuberculin was defined in 1952 as the activity contained in a specified weight of the International Standard for PPD of Mammalian Tuberculin (Ref. 2).

Only dilutions of Seibert's lot 49608 can be called PPD–S. All other PPD's should be identified as PPD with lot number and producer. The standard test dose, 5 TU, refers to the equivalence of biological activity of a PPD preparation with that contained in 5 TU of PPD–S dissolved in Seibert's phosphate buffer. When diluted in a buffered diluent, tuberculoprotein is adsorbed in varying amounts to glass and plastic. Recently, small amounts of the detergent polysorbate 80 (Tween–80®) have been added to the diluent to reduce adsorption. Five TU of PPD with polysorbate 80 may contain a smaller quantity of protein than 5 TU of PPD–S (which does not contain detergent); however, the biological activity contained in 5 TU of the PPD with detergent, or any other PPD, must be, by definition, equivalent to the activity contained in 5 TU of PPD–S. Polysorbate 80 stabilized solutions of PPD labeled as 1 TU and 250 TU are available; however, these potencies are derived by calculation from the dosage determined to be 5 TU. Clinical evaluation (biologic equivalency in humans) has not been required. Injection of Tuberculin, PPD into the skin produces a localized reaction in sensitized persons. Tuberculin, PPD is administered intracutaneously by the Mantoux method and by multiple puncture techniques. The reaction to injected PPD in sensitized individuals consists of an area of induration (usually with surrounding erythema) which varies in size and intensity according to the dosage of tuberculin and the sensitivity of the individual. Skin reactions to PPD may occur in individuals infected with mycobacteria other than *M. tuberculosis* and *M. bovis*.

A positive tuberculin test usually develops 2 to 10 weeks after initial infection with *M. tuberculosis* or *M. bovis*. Once acquired, sensitivity to tuberculin tends to persist throughout life. It may wane with advancing age or disappear if treatment of the infection is given in its earliest stages. Sensitivity may decrease or disappear temporarily during a variety of intercurrent illnesses, during treatment with adrenal corticosteroids, and in tuberculous patients who are very sick (Refs. 3 through 11). When the usual causes for negative reactions are excluded, failure to react to a tuberculin test is helpful in excluding the diagnosis of tuberculosis.

(a) *Effectiveness.* Practically all of the valid data in the United States on sensitivity and specificity of tuberculin have been obtained from studies using PPD bioequivalent to 5 TU of PPD–S. While there is a paucity of data relating to the sensitivity and specificity of the 250 TU strength, additional information may be inferred about concentrated PPD from studies employing 100 TU (Refs. 12 through 15). Apparently, increased sensitivity can be obtained only at the cost of decreased specificity (Ref. 16). Sensitivity and specificity of PPD may be affected by recently recognized problems of standardization: preparations found to be equivalent in guinea pigs may not be equivalent in humans (Refs. 17 through 19). The effectiveness may also be impaired by the known propensity of PPD to become absorbed to surfaces.

(1) *Sensitivity.* Tuberculin possesses excellent, although not perfect, ability to identify all individuals with specific

immunologic experience. Virtually all tuberculous patients will react to the intracutaneous injection of tuberculin if a sufficiently strong dose is used. A few patients will not react at all (Refs. 16, 20 through 22).

The intensity of the reaction may be diminished by many factors which affect delayed hypersensitivity reactions in a nonspecific manner by mechanisms not fully understood (Ref. 3). These factors or conditions include acute virus infections or vaccination with live virus vaccines; immunosuppression by disease, drugs, or steriod hormones; a state of general anergy such as that associated with sarcoidosis or malignancy, especially lymphoma; malnutrition, especially in children; overwhelming infection; and the waning of delayed hypersensitivity associated with advancing age (Refs. 4 through 11). In addition, there is a small proportion of individuals with tuberculosis who have none of the above conditions but who still do not react to ordinary doses of tuberculin. Although a recent publication suggests that this proportion may be as high as 20 percent (Ref. 23), observations over many years have documented that it is usually no more than 5 percent (occasionally up to 10 percent) with the use of intermediate strength tuberculin (5 TU), and 1 percent or less with high doses (100 to 250 TU) of tuberculin (Refs. 20 through 22, 24). In most patients who are very sick with tuberculosis, the tuberculin test, when previously negative, becomes positive after a few weeks of treatment.

(2) *Specificity.* Although the tuberculin test is very good for the recognition of tuberculous infection, infections with mycobacteria other than *M. tuberculosis* or *M. bovis* can cause reactions to tuberculin (Refs. 25 through 27). This not surprising since all mycobacteria and many nocardia species share antigens (Refs. 28 through 33). The specificity of the PPD test varies geographically according to the prevalence of these other infections, specificity generally being highest in the Arctic and at high altitudes and lowest in the tropics and at low elevations (Refs. 34 and 35).

Specificity is intimately related to the concentration of tuberculin used for the test. Furcolow et al. showed that most individuals, including infants, reacted to some doses of PPD when increasing amounts were given (Ref. 16). A larger proportion of persons will react to high-dose tuberculin than to low or intermediate dose tuberculin. Some of these individuals have been infected with *M. tuberculosis*, some with other species of mycobacteria, and some probably with other organisms that share antigens in the cell wall, such as nocardia and corynebacteria (Refs. 28 through 33). The main value of a high-dose tuberculin test is in a negative reaction, which makes a diagnosis of mycobacterial infection unlikely in the absence of the conditions listed above which diminish all delayed hypersensitivity reactions.

"Conversions" to positive tuberculin tests may sometimes result from the "booster" effect of a previous test (Refs. 36 through 39) and after blood transfusion from a tuberculin positive donor (Ref. 40). Conversion after blood transfusion presumably is due to the transfer of sensitized lymphocytes or their products. The tuberculin test has remained positive in a recipient for at least 8 months (Ref. 41). Reversion of a positive test to negative also has been documented (Refs. 42 through 44). This is most apt to occur when isoniazid treatment is given shortly after infection has occurred.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material*—(1) *Chemical composition.* Extensive investigation by increasingly sophisticated chemical and immunologic techniques has resulted in a wealth of information on the chemical composition of Tuberculin, PPD. It is agreed that the major active principles are protein in nature. Seibert found that PPD–S contained 1.2 percent nucleic acid and 5.9 percent polysaccharide (Ref. 45). She was able to isolate two distinct polysaccharide fractions (I and II) and three distinguishable protein fractions (A, B, and C) (Ref. 46). The A protein fraction demonstrated the slowest migration when studied by electrophoresis. While the mobility of the B and C protein fractions was approximately the same, their solubility properties were different. Protein A was more potent and possibly more specific than protein C in eliciting the tuberculin reaction in infected animals and human beings. Polysaccharide fraction I was a small molecule and polysaccharide fraction II was a large molecule. Neither of these polysaccharide fractions produced skin reactions in tuberculous animals or human beings. Daniel and Affronti (Ref. 47) recently have identified Seibert's three protein fractions in an immuno-electrophoretic system with the U.S.-Japan reference anti-serum. Also applying immunoelectrophoresis to whole PPD, (not PPD–S), Moulton et al. (Ref. 48) identified five comopnents, of which only three appeared to be specific as identified by cross-sensitivity testing in guinea pigs. Protein was again implicated as the reactive component when treatment with pronase destroyed the antigenic reactivity of these three components. Daniel and Ferguson described two purified protein fractions which they called a_1 and a_2 (Ref. 49), while Nagai and his co-workers described five relatively pure proteins each with a molecular weight of approximately 10,000 daltons (Ref. 50).

(2) *Reproducibility.* It has long been recognized that potency can vary from batch to batch. Seibert (Ref. 46), in evaluating the production of tuberculin, found that four different strains of tubercle bacilli yielded different relative amounts of polysaccharide fractions I and II and protein fractions A, B, and C. Further, different batches of tuberculin prepared from the same strains yielded different relative amounts of these five products.

Nagai et al. (Ref. 50) demonstrated that protein content of the culture filtrate increases with the age of the culture. Using disc gel analysis, their work showed that sharp protein filtrate fractions can be identified between the 3d and 5th week of culture growth, but thereafter protein modifications make identification more difficult.

(3) *Standardization.* Standardization in an animal model may not reflect the situation in other animals or humans (Refs. 17 through 19). However, following batch standardization in human populations (Ref. 51), it is possible to standardize PPD lots against the Reference Standard (PPD–S) using sensitized guinea pigs, provided an adequate number of animals is used and readings are made in a fashion that obviates reader bias (Refs. 52 and 53). Recently, a new method of comparing tuberculin preparations by using lymphocyte cultures has been described but not yet evaluated (Ref. 54).

(4) *Stability.* PPD is stable for long periods under proper conditions of storage. Inactivation by ultraviolet light and removal by adsorption to glass and plastic has been documented (Refs. 55 through 57). Loss of activity by adsorption has been diminished considerably by the addition of a wetting agent such as polysorbate 80 (Refs. 58 through 60).

(5) *Sensitization.* In the ordinarily used dosages, PPD has not been shown to induce a primary immunologic response. Tukey, DeFour and Seibert (Ref. 61) demonstrated that repeated injections of PPD given over periods of weeks and months to student nurses who failed to react to high dose PPD initially, did not produce positive skin reactions. Seibert reported (Ref. 62) that rabbits could be sensitized by repeated injections of 10 mg of PPD, whereas nine injections of 0.05 mg did not sensitize any of six normal guinea pigs.

It has not been demonstrated that PPD augments the immunologic response except in persons whose sensitivity has waned and is "boosted" by a tuberculin test (Refs. 36 through 39).

(6) *Elimination by host.* Although the clearance of PPD from the skin test site within 24 hours has been demonstrated in guinea pigs (Refs. 63 and 64), no data are available on its elimination from the human body.

(7) *Diluent.* The currently used diluents are not sensitizing. In animal and human studies performed at the World Health Organization Tuberculosis Research Office in Copenhagen, no sensitization could be induced by polysorbate 80 alone (Ref. 58).

(ii) *Test method*—(1) *Application.* PPD is applied by the intracutaneous (Mantoux) or multiple puncture technique. The Mantoux test is simple and accurate. The multiple puncture tests are simple but deliver a variable and undetermined quantity of test material into the skin.

(2) *Measurement.* The reaction to these tests is a roughly circular area of induration, usually with clear-cut boundaries. The Mantoux test is easily readable by trained observers. The multiple puncture methods may result in one or

several small reactions which may coalesce to form a circular reaction.

(3) *Quantitative dose response.* The Mantoux method produces a range of reactions that is sufficient for dose-response studies. These studies are more difficult with the multiple puncture methods because of the discontinuous distribution of reaction sizes resulting from coalescence of multiple small reactions.

(4) *Duration of reaction.* In a study of reactions caused by PPD, it was found that mean induration size was relatively constant from 24 to 120 hours (Ref. 65).

(b) *Safety*—(i) *Host response.* PPD has had widespread use in millions of persons over many years, with great safety. There is no evidence that it produces anaphylaxis; carcinogenic, teratogenic, or mutagenic effects; sensitization to the product; reactivation of tuberculosis; or interference with other medications. Infrequently, there may be a severe local skin reaction with vesiculation, pain, and tissue necrosis. Very severe reactions may be associated with constitutional symptoms of fever and malaise as well as lymphangitis and lymphadenitis, but such reactions are rare. Localized scarring and change in skin pigmentation may occur infrequently following severe reactions; however, very little residual scarring was noted from the reactions produced by inadvertent injection of 10,000 TU (Ref. 66).

(ii) *Test material*—(1) *Sterility.* The method of preparation can be expected to kill all microbial agents. The standard sterility tests seem to be sufficient for this particular substance.

(2) *Purity.* It is highly unlikely that any dead microbial agents are present. No tests are done for non-microbial contaminants. Other contaminants are highly unlikely.

(3) *Elimination by host.* No data are available.

(4) *Toxicity.* There is no indication of toxicity when PPD is used intracutaneously in the usual dosages. Tuberculoprotein has been shown to have a low-grade intrinsic toxicity for non-sensitized animals. Seibert and Munday found that normal animals could be killed by the injection of PPD, but it required 50 times the amount lethal for infected animals (Ref. 67). The standard required safety tests should be sufficient to protect against the inadvertent introduction of toxic materials.

(iii) *Test method.* The intracutaneous Mantoux and multiple puncture methods of injection produce very little pain or bleeding, and are essentially noninjurious.

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Tuberculin, PPD (Diluted) Aplisol[R] By Parke-Davis

1. *Product.* Tuberculin, PPD (Diluted) Aplisol[R] manufactured by Parke, Davis and Co.

2. *Description.* The primary purpose of the Parke, Davis and Company Tuberculin, PPD product is to detect delayed hypersensitivity in individuals who have had immunologic experience with *Mycobacterium tuberculosis* and *Mycobacterium bovis*. The product is an aqueous solution of a purified protein derivative of tuberculin (PPD). It was prepared by the ammonium sulphate method of Seibert (Ref. 1) from culture filtrates of *M. tuberculosis*. The final solution is stabilized with polysorbate 80 (Tween–80[R]), is buffered with potassium and sodium phosphate (pH 7.3 plus or minus 0.1), contains about 0.35 percent phenol as a preservative, and is bioequivalent in potency to the standard PPD–S (5 TU/0.1 ml). The marketed product, injected by the Mantoux method, is ready for use without dilution. At the present time all production lots are prepared from a single, lyophilized Master "Batch" Rx 974777 which has been clinically standardized in order to "eliminate lot-to-lot variation."

Master Batch Rx 974777 was prepared from the culture filtrate of *M. tuberculosis* grown as mats or surface pellicles in Long's liquid synthetic medium (Ref. 2). At the end of incubation, the flasks were heated for 3 hours in flowing steam, the larger particles removed by filtration through coarse filter paper, and the various filtrates were pooled. Phenol was added to a final concentration of 0.5 percent and the filtrates passed through a Mandler candle or membrane filter. After tests for sterility, safety, and content of total protein nitrogen, carbohydrate, and nucleic acid, the filtrate was washed free of medium constituents "until chloride negative." The filtrate was then reduced in volume to a standard protein concentration. Saturated ammonium sulphate was added to the concentrated filtrate at pH 7.0 to 7.4. The resultant precipitate was recovered by centrifugation and dissolved in phosphate buffer, pH 7.2 to 7.6 followed by precipitation with ammonium sulphate and resolution in phosphate buffer. The final precipitate was dissolved in phosphate buffer pH 7.2 to 7.6 containing 0.5 percent phenol. This final solution was exhaustively washed by ultrafiltration with the phosphate buffer-phenol diluent until it was free of sulphate ions. Microkjeldahl nitrogen determinations were performed on the final solution and protein content was calculated on the basis of 16.3 percent nitrogen in PPD. The final solution was distributed into vials, lyophilized and stored at 4° C.

The Parke-Davis Tuberculin, PPD (bioequivalent to 5 TU PPD–S) is prepared from a concentrate of PPD made by reconstituting portions of the lyophilized Master Batch Rx 974777 in sodium and potassium phosphate buffered saline containing 0.0005 percent polysorbate 80 and approximately 0.35 percent phenol to make a predetermined fixed final volume on the basis of previous standardization. After sterilization by membrane filtration, the concentrate is assayed for total nitrogen (protein determined on the basis of 16.3 percent nitrogen in PPD), nucleic acid, and carbohydrate, and tested for sterility. Finally, the potency is estimated in guinea pigs (250,000 TU plus or minus 62,500 TU/ml). The final product is made by diluting this concentrate in the appropriate buffer to a predetermined, fixed volume and then subjecting it to the following assays: Phenol content, polysorbate content, sterility test, and potency test in guinea pigs. The bulk PPD diluted solution is then aseptically filled into presterilized vials and stoppered.

3. *Analysis*—(a) *Effectiveness.* In their submitted material, Parke, Davis and Company report collated data from five well controlled studies in humans using Tuberculin, PPD diluted Aplisol and Tuberculin, PPD–S. The sensitivity and specificity of this product were shown to be identical with that of PPD–S.

There was little information specifically referring to the ability of this product to identify all individuals who have had immunologic experience with *M. tuberculosis*. Cross-reactions in guinea pigs do not occur between the Parke, Davis and Company Tuberculin, PPD

and coccidioidin, histoplasmin, or blastomycin.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material*—(1) *Composition.* The chemical composition of the immunologically active material is not defined. The product is characterized according to its nucleic acid, carbohydrate and nitrogen content. The diluent contains sodium and potassium phosphate, sodium chloride, polysorbate 80 and phenol. The pH of the buffered diluent is specified to be 7.3 plus or minus 0.1. All of the chemicals are reagent grade except for polysorbate 80 which is USP grade.

(2) *Reproducibility.* The Parke-Davis product is made from a single Master Batch Rx 974777 which should eliminate lot-to-lot variation with the exception of possible batch deterioration. In the company protocol 123–12 two "working concentrations", T4986 and T5664, were tested against each other and against PPD–S in well-controlled studies in humans. The two lots were found to be equally potent to each other and to PPD–S.

(3) *Standardization in laboratory systems.* The potency of Parke, Davis and Company Tuberculin, PPD is estimated in guinea pigs by comparing the skin test reaction of the lot under test with that of the U.S. Tuberculin, PPD–S standard. For the guinea pig potency tests each lot is compared with standard tuberculin in animals sensitized by the injection of the heat-killed strain Jamaica 22 *M. tuberculosis* in mineral oil. The prescribed test calls for at least four animals, each of which is tested with four different concentrations of the test material and standard. The potency is estimated by a "computer program." The guinea pig test is used for "in-house manufacturing control" only. The standard of acceptability for the final product consists of potency of 16 TU plus or minus 3.2 TU per 0.1 ml (20 percent variation). It is stated that results of replicate potency determinations on three consecutive production lots were submitted to the Bureau of Biologics.

(4) *Stability.* The manufacturer states "stability data on the first five lots of Aplisol are being provided to the Bureau of Biologics as the lots become available." While no data were submitted, it is stated that potency tests are performed on final container samples stored at 4, 22, 37, and 45° C over a period of at least 30 months. In clinical trials of lot stability performed on inmates of two different Michigan institutions (410 volunteers) with test preparations X–42432 (5 TU/0.1 ml 5 ppm polysorbate 80), X–42431 (5 TU/0.1 ml 15 ppm polysorbate 80), and T–5983 (5 TU/0.1 ml) the following were determined:

(a) The PPD potency was unchanged throughout the use life of stabilized 50 dose vials of their product.

(b) Polysorbate 80 at a concentration of 15 ppm was sufficient to maintain the potency in a secondary container (glass syringe) for at least 8 days.

(c) Two different "working concentrates" from the Master Batch containing 5 ppm polysorbate 80 were equipotent with 5 TU PPD–S in biological activity.

(d) A concentration of 5 ppm polysorbate 80 was insufficient to maintain potency of their product in a secondary container (glass syringe) for a least 8 days.

These conclusions were all drawn from Protocol 123–21 which was included in the material submitted by Parke, Davis and Company.

(5) *Sensitization.* Cross sensitization studies have been performed at Parke, Davis and Company using guinea pigs which have been sensitized with "Tuberculin, Histoplasmin, Blastomycin". In each instance, the homologous test antigen elicited responses equivalent to the homologous standard while the heterologous test antigen elicited little or no response. There appear to be no specific data with regard to sensitization of humans after repeated inoculations of their specific product.

(6) *Elimination by the host.* There were no data provided regarding elimination of this particular product by the host.

(7) *Diluent.* The diluent is checked for pH, conductivity, and phenol concentration. No reactions have been reported from the use of the diluent. Phenol, 0.35 percent, would appear to be non-sensitizing based on extensive clinical experience with many products into which it is incorporated. In animal studies performed at the World Health Organization Tuberculosis Research Office in Copenhagen (Ref. 3), employing guinea pigs and rabbits, no sensitization could be induced against polysorbate 80 or against the combination of polysorbate and tuberculin. Similar results were also obtained in man.

(ii) *Test method*—(1) *Application and dosage.* The application of the test (Mantoux method) is simple and accurate, involving the use of a tuberculin syringe and needle.

(2) *Quantitative dose response and duration of reaction.* The manufacturer suggests that reactions be read at 48 to 72 hours in man and at 18 to 24 hours in experimental guinea pig systems. No specific information was available in regard to a defined time course for reactions with this product. In clinical studies of the quantitative relationships between the Parke-Davis product and PPD–S skin test responses, the mean response increased as expected with increasing concentrations over a test dose range of three concentrations (1:3:9 with the middle dilution containing, based on preliminary clinical assay, a level of PPD approximately equal in potency to 5 TU of PPD–S). These data were presented in Protocols 123–9 and 123–10.

(b) *Safety*—(i) *Host Response.* In clinical tests in over 1,700 volunteers in the Forida and Michigan areas to evaluate potency and stability of the product, no constitutional or anaphylactic reactions were reported. The manufacturer lists several million doses sold in the past years in 10 and 50 dose vials. They state since its introduction that "there have been only three complaints of local reactions, three false positives and one false negative reported in letters and telephone calls from medical personnel." They further state that none of these reactions were serious. The validity of these complaints has never been established and the actual type of reactions was not presented. The Panel found no information on potental carcinogenic, teratogenic, or mutagenic effects from this product. As in any tuberculin test, severe skin reactions can occur occasionally with vesiculation and necrosis. In some of these, lymphangitis, lymphadenitis, and symptoms of malaise and fever may occur for a day or so. Rarely in severe reactions, scarring and changes in pigmentation may occur.

(ii) *Test material*—(1) *Sterility.* The manufacturer states that Master Batch Rx 974777 has been tested for sterility and safety tests in guinea pigs at various stages in the processing procedure. These tests are conducted in a manner to comply with §§ 610.12 and 650.13(b) of the Federal regulations. There appear to be no routine tests performed for the presence of viral, mycoplasmal, or fungal contamination. It is highly unlikely that such contaminants would be present.

(2) *Purity.* Other contaminants are unlikely.

(3) *Elimination by host.* There was no information on product elimination by the host.

(4) *Toxicity.* This product appears to be nontoxic by human experience.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious. The small amount of polysorbate 80 detergent (5 ppm) added to the solution does not cause an irritant or painful response.

4. *Recommendations.* (a) The Panel recommends that this product should be placed in Category I, safe and effective. For future batches, the manufacturer should submit acceptable data based on scientifically sound methods to demonstrate effectiveness in humans (Ref. 4). These data should include for each batch:

(i) Comparison of the product with the standard by means of dose response curves.

(ii) Distribution of reaction sizes in persons known to be infected and in those presumed to be uninfected with *M. tuberculosis* and in addition in persons presumed to be infected with other mycobacteria.

(iii) Chemical characterization (including protein, carbohydrate, lipid and nucleic acid) should be performed on each batch to assess consistency of production.

(b) Furthermore, the Panel recommended no changes in the labeling.

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TUBERCULIN, PPD, MULTIPLE PUNCTURE DEVICE (APLITEST) BY PARKE-DAVIS

1. *Product.* Tuberculin, PPD, Multiple Puncture Device (Aplitest) manufactured by Parke, Davis and Company.

2. *Description.* The Parke, Davis and Company multiple puncture device, Aplitest, is a "screening tool indicated to detect tuberculin-sensitive individuals." The manufacturer states (in the package insert) that the test unit has "been standardized by clinical studies in human subjects to give reactions equivalent to 5 TU of PPD–S administered intradermally in the Mantoux test" and labels the product "each device is clinically equivalent in potency to 5 TU administered intradermally."

Master Batch No. 975302 Tuberculin PPD used for the Aplitest was prepared from the D.T. strain of human tubercle bacilli. The organisms are inoculated into L-asparagine synthetic medium.

Flasks showing good growth are processed through a series of steps until the final precipitation with ammonium sulfate. The protein nitrogen content is determined. The final solution is then filled into vials, frozen and dried in a vacuum dryer at not more than 30° C.

Aplitest (tuberculin, PPD, concentrated) is a single-use multiple puncture device, consisting of a cylindrical plastic holder, open on one end with four stainless steel tines on the other end. The tines are coated with concentrated, dried PPD. The devices are designed so that the narrow tine-bearing end of each one fits into the hollow end of an adjacent unit, or into a protective cap. After the multiple puncture units are assembled, they are cleaned and degreased. The tines are machine-dipped in the PPD solution and then dried. The devices are machine-packed and this final product is sterilized.

3. *Analysis*—(a) *Effectiveness.* All reported studies on the sensitivity and specificity of this product used Lot No. X–42194 prepared from a lyophilized Master Batch No. 975302 PPD powder "which is reserved for all preparations of Tuberculin, PPD, Multiple Puncture Devices." Lot No. X–42194 was not intended for commercial distribution; subsequent lot preparations to be used in market sales depend upon "weighted mean potency" calculations arising from a 4 x 4 block comparison on 4 guinea pigs as described under the unit dealing with reproducibility.

Lot No. X–42194 was used in six studies conducted by the manufacturer. The device to be marketed (containing 0.2 micrograms of PPD/device) was tested for co-positivity and co-negativity with 5 TU of PPD–S given by the Mantoux technique on 1,686 persons, of whom 372 reacted to the standard Mantoux with 10 or more mm of induration. However, the manufacturer excluded the results of 603 tests, including 212 Mantoux reactors, on the grounds that the simultaneous administration of three multiple puncture tests and one Mantoux test (for a maximum possible dose of 75 TU) would somehow overload the host response mechanisms and thereby render the results invalid.

A summary report of the four studies considered by the manufacturer to be valid gives the combined results on 1,083 subjects at four locations in Michigan, Florida, and the Bahama Islands. The following table gives the results of these four studies, both combined, as in the summary report, and separately because there is reason to believe that combination in this instance is inappropriate. The table also gives results from the two sides excluded from the summary report. If a positive Aplitest result is defined as 2 or more mm of induration, and a positive Mantoux result as 10 or more mm of induration, the following summary statistics are obtained. In all cases, the averages of multiple readings were used.

Population	Number	Percent of copositivity	Percent of conegativity	Percent of agreement [1]	Percent of false positives [2]
Summary of 4	1,083	97	82	79	52
Fort Myers, Fla	358	99	82	81	68
Ypsilanti, Mich	143	87	82	69	63
Pontiac, Mich	248	99	85	84	19
Bahamas	334	92	81	73	78
Excluded by the manufacturer:					
Florida prisons	215	97	42	39	37
Do	388	92	70	62	50

[1] Estimated by Youden's J statistic (ref. 1).
[2] Positive Aplitest and negative Mantoux among total persons positive to Aplitest.

In the package insert the manufacturer recommends following the American Thoracic Society guidelines on interpreting the skin test response to multiple puncture tests:

2 mm or more of induration without coalescense—Doubtful Reaction. Reactions of this size range reflect sensitivity that can result from infection with either atypical mycobacteria or *M. tuberculosis:* hence they are classified as doubtful. A standard Mantoux test should be done on all subjects in this group * * *.

In all of the studies reported by the manufacturer, persons with vesiculation to the Aplitest had 10 mm or more of induration to 5 TU PPD by the Mantoux method.

Study No.	—14	14A	15	16	17	19	Totals
Mantoux positive (10 mm).	2.5	18.7	4	2	16	1	44.2
Tine vesiculation	2.5	19.0	4	2	16	1	44.5

Of the 200 persons with a coalescent reaction to the Aplitest but without vesiculation, 86 percent were positive (10 mm) PPD 5 TU Mantoux reactors.

Study No.	14	14A	15	16	17	19	Totals
Mantoux Positive (10 mm).	62.0	88.3	13.3	6.7	48.7	3.0	172
Tine coalescence	66.5	55.3	17.9	6.7	51.0	3.5	200

The manufacturer's package insert recommends that "vesiculation" or "coalescence of induration from two or more puncture points" be considered as "positive reaction—the test may be interpreted as positive and equivalent to a reaction of 10 mm or more of induration with PPD–S (5 TU) administered by Mantoux test."

CHARACTERISTICS OF EFFECTIVENESS

(1) *Test material*—(1) *Chemical composition.* The PPD for the Aplitest devices was characterized only in general terms. It is analyzed for nitrogen, nucleic acid, and carbohydrate content. Chlorides and sulfates were removed in the course of preparation. The diluent for the final dipping solution contains sodium and potassium phosphates, sodium citrate, and phenol. Each device is estimated to contain 0.1 micrograms of phenol.

(2) *Reproducibility.* To date, only one lot (No. X–42194) of Aplitest devices with the current dosage has been prepared from the large Master Batch No. 975302. The manufacturer states that future lots for the Aplitest device will be prepared by dissolving a measured amount of PPD Batch No. 975302 in sterile buffered diluent containing approximately 0.5 percent phenol to a "pre-determined concentration"—apparently based on the weight/volume ratio found to be satisfactory for clinical use during the human studies with Lot No. X–42194 0.2 micrograms/device. A sample of this dip solution is assayed for guinea pig potency (4 sensitized guinea pigs) and must meet a standard of 125,000 plus or minus 62,500 TU/ml. Apparently, the tests are not applied in a blind or random manner and no precautions are taken to avoid reader bias.

The solution is stored at 2 to 8° C while this test is satisfactorily completed and then used for tine dipping.

(3) *Standardization.* The final product has apparently been standardized in guinea pigs. The manufacturer states that the tines from "at least 32 devices" are eluted in 2.0 cc of phosphate buffer. This provides "an eluate containing at least (sic) 40 TU per ml." "The eluate is assayed for potency in guinea pigs in accordance with Regulations." "Because the claimed potency has been shown to be clinically equivalent to 5 TU of PPD–S (Mantoux) by comparison of human response data the potency estimate is used as laboratory confirmation of potency.

The acceptance standard for Tuberculin, PPD, Aplitest prepared from Tuberculin PPD Master Lot No. 975302 is 2.5 TU (plus or minus 50 percent) per device in guinea pigs."

It should be emphasized that the term TU is used as a potency estimate for each tuberculin produced by the manufacturer and serves only as a measurement factor in comparing a product to 5 TU PPD–S. With regard to this product the manufacturer states that 0.2 micrograms of PPD Batch number 975302 produces a reaction comparable to 5 TU PPD–S (Mantoux).

In addition to the clinical trials of Aplitest devices noted under "Effectiveness," lot No. X–42194 has been standardized in the guinea pig against "U.S. Standard PPD No. 76."

To prepare dip solution Lot X–42194, a solution was used that had an actual potency of 2.7 mg of PPD, or 135,000 TU, per ml. Potency tests on the final product were made by clipping the tips from 30 devices and eluting them in 2 ml of phosphate buffer. The resulting solution should contain 40 TU per ml. This material was used to skin test 4 sensitized guinea pigs and the reactions were compared with those produced by 40 TU per ml of U.S. Standard PPD No. 76 using 4 dilutions of each preparation. The estimated potency for this lot was 2.52 TU per device. Thus, 2.5 TU/device is the manufacturer's standard for future lot productions from Master Batch No. 975302.

Apparently the guinea pig skin tests were not applied in a blind or random manner and no precautions were taken to avoid reader bias.

(4) *Stability*. Dip solution Lot No. X–42194 studied by the guinea pig potency test was found to be stable for 15 months when stored at 20 to 25° C.

Comparisons of human tests of the currently used lot (X–42194) with 5 TU of PPD–S over an 11-month period have given no indication of any change in potency. However, the number of reactors in these tests is too small to detect any but gross changes. It seems likely that dried concentrated PPD will be stable. The manufacturer recommends that it be kept below 30° C (86° F).

(5) *Sensitization*. No specific data were given regarding the induction or augmentation of immunologic response from this material. Principles described in generic review should apply to this product.

(6) *Elimination by host*. No data were provided.

(7) *Diluent*. The product is administered in the dried state.

(ii) *Test method*—(1) *Application*. When a test is to be given, the times are exposed by removing a protective cap or an adjacent device. The tines are then applied firmly to the cleansed taut skin (usualy of the volar surface of the forearm) for approximately one second. The amount of tuberculin deposited in the skin has not been determined and probably cannot be controlled except within broad limits.

(2) *Measurement of test*. The manufactuer recommends that the reactions be read in 48 to 72 hours by measuring the diameter of the largest area of induration. The induration produced has a reasonably clear-cut boundary. On the basis of limited experience the manufacturer advises that reactions showing either vesiculation or coalescence be considered as positive without rechecking with a standard Mantoux tuberculin test.

(3) *Quantitative dose response*. The manufactuer's submitted study protocol 123–14 allowed a comparison of the reactions to tine devices carrying 0.2, 0.4, and 0.8 micrograms of PPD per device. An analysis of these data provides the following comparisons:

	0.2 mcg versus PPD-S	0.4 mcg versus PPD-S	0.8 mcg versus PPD-S
Correlation coefficient	0.67	0.63	0.61
SE, correlation coefficient	.04	.04	.04
Slope of regression line	.54	.52	.51
SE, slope	.042	.044	.046
Percent vesicular	1.2	2.6	3.7
SE, percent	.74	1.1	1.3

There are no significant differences between any of the correlation coefficients, slopes, or percentages with vesicular reactions. The calculations for correlation coefficients and slopes are based on the data as grouped by the manufacturer. Arbitrary values are assigned to each category: 0, 1, and 2 are used for the PPD–S categories and 0, 1, 2, and 3 for the Aplitest categories. While this procedure clearly introduces errors, they should be essentially the same for each of the three tables and hence cancel out when comparing correlation coefficients or regression lines. The data indicate that when products employ high concentrations of tuberculin, exact dosage seems to matter very little, a four-fold increase in the PPD concentration of this product causing no demonstrable difference in reactions.

(4) *Duration of reaction*. No data were provided.

(b) *Safety*.—(i) *Host response*. There is no reason to suspect that this product would differ from other tuberculins with regard to anaphylaxis, carcinogenic effects, teratogenic effects, mutagenic effects, sensitization to product, reactivation of disease or interference with the action of other medications. In reviewing the data provided by the manufacturer, among 372 reactors to the standard Mantoux test, there were 46 persons who had vesicular reactions to the Aplitest. No other data were given regarding long- or short-term complications, except that the reported studies on 1,675 persons mention no complications, implying thereby that none occurred.

(ii) *Test material*.—(1) *Sterility*. After initial filtration, the batch is tested for the presence of living tubercle bacilli by injecting 1 ml into each of four guinea pigs. At least three must live for 6 weeks and none can show evidence of tuberculosis at necropsy.

The manufacturer states that it intends to prepare a sterile stock PPD solution (lot) and remove a separate aliquot for each day's use, discarding the unused portion. The portion used for the day's dipping is kept at 5° C during the process and is preserved with aproximately 0.5 percent phenol. The dipped tines are terminally sterilized using ethylene oxide. The dip test solution used each day is tested for sterility.

The required sterility tests on the final product are accomplished by removing the metal tine rings from the plastic handles of 20 Aplitest devices. Each ring is added to a tube of fluid thioglycollate medium. Twenty additional rings are similarly incubated in tubes of soybean-casein digest medium. It is not likely that there would be contamination with viral, mycoplasmal or fungal agents.

(2) *Purity*. No data were provided except that only 0.1 mcg of phenol is calculated to be present on a single device. Other contaminants are unlikely.

(3) *Elimination by host*. No data were provided.

(4) *Toxicity*. No mention was made of any toxic effects noted during the testing of approximately 2,000 persons of all ages.

The standard safety tests in two guinea pigs and two mice are performed with the material eluted from 12 devices in 12 ml of saline. Five ml of this solution is injected subcutaneously (sic) into each of two ginea pigs. Each of two adult mice is inoculated subcutaneously (sic) with 0.5 ml of the eluate.

NOTE.—The recommended parenteral route is now an intraperitoneal injection. The animals are then observed for 7 days for "freedom from significant signs or death."

(iii) *Test method*. The test produces minimal momentary pain. Although 90 of 394 subjects had bleeding after being tested, this occurred at the site of only one tine puncture in 75 subjects. The test is essentially noninjurious.

4. *Recommendations*. The Panel recommends that this product should be placed in Category I because the present product has been demonstrated to be safe and effective. Since there is ample evidence in the literature (see the Generic Statement) that batches of tuberculin may vary widely in potency, it is necessary that each new batch be tested for potency (sensitivity) in infected humans. Potency tests in guinea pigs do not necessarily reflect potency in human beings. Since the test is to be used for screening purposes, it is necessary to test only for sensitivity and not specificity. The Panel recommends that sensitivity be sufficient to elicit positive reactions in at least 50 persons who are known to have had bacteriologically confirmed tuberculosis and who are tuberculin positive as demonstrated by a simultaneous Mantoux test with 5 TU of PPD.

(a) Quality control. Because of the possibility that the mechanical dipping process may occasionally fail to deposit tuberculin on the tines of an individual unit, routine testing of the product should include a test for protein or other test material on the tines of randomly selected individual units.

(b) Labeling. (i) Delete references implying equivalent significance of reactions to this product and to those of 5

TU of Tuberculin, PPD by the Mantoux (intracutaneous) method.

(ii) Statements of the interpretation of the test should be consistent with the current guidelines published by the American Thoracic Society (because the company's data do not adequately demonstrate that coalescence is equivalent to a positive test with 5 TU of Tuberculin, PPD by the Mantoux method).

(c) Advertising. (1) Delete statement suggesting that PPD as used in this device is more specific than Old Tuberculin.

(ii) Delete "Provides savings in time, effort and money by elimination of retesting."

REFERENCE

(1) Youden, W. J., "Index for Rating Diagnostic Tests," *Cancer* 3:32–35, 1950.

TUBERCULIN, PPD (MANTOUX) BY CONNAUGHT

1. *Product.* Tuberculin, PPD (Mantoux) manufactured by Connaught Laboratories Ltd.

2. *Description.* The primary purpose of the Connaught Laboratories Ltd. Tuberculin, Purified Protein product is to detect delayed hypersensitivity in individuals who have had immunologic experience with *Mycobacterium tuberculosis* or *Mycobacterium bovis*.

The Connaught product was purified by precipitation from the culture filtrate derived from the L-asparagine medium on which the strain "Johnston" of *M. tuberculosis* had grown as a surface pellicle. The stock concentrate was dissolved in phosphate buffered saline containing 0.5 percent phenol. The potency of this stock concentrate was checked in eight suitably sensitized guinea pigs using two dose levels of test material and PPD–S. A large batch was produced in order to minimize lot-to-lot variation. This Master Batch CT68 was made from 10 individual lots of stock concentrate which were selected and pooled. The final product, diluted in phosphate buffered saline containing 0.3 percent phenol as a preservative and 5 ppm polysorbate-80 (Tween-80R) as a stabilizer, is marketed in three strengths, 1 TU, 5 TU, and 250 TU.

3. *Analysis.*—(a) *Effectiveness*—(i) *Sensitivity.* Connaught CT68 identifies tuberculous infected individuals as well as PPD–S does. This was demonstrated conclusively in studies under the direction of Dr. G. Wijsmuller in which batch CT68 was compared with the Center for Disease Control standard PPD–S in adequate numbers of humans.

(ii) *Specificity.* (See the discussion in the Tuberculin, PPD, Generic Statement.) It is not unreasonable to assume that batch CT68 also will show cross reactions in individuals who have been infected with other mycobacteria. One study by Dr. Wijsmuller in guinea pigs tends to confirm this assumption. In addition, in an identity test performed on one vial of the final container of each lot, the material must produce no reaction in a normal guinea pig, and a larger reaction in a guinea pig sensitized with *M. tuberculosis* than in a guinea pig sensitized with *M. avium-intracellulare*. Reactions are compared with those produced by standard PPD.

CHARACTERISTICS OF EFFECTIVENESS

(i) *Test material.*—(1) *Chemical composition.* The product is the 2 percent trichloroacetic acid precipitable fraction of a pooled lot of culture filtrate. The nucleic acid content is approximately 5 percent. The only other information on the chemical composition is the nitrogen content as measured by the Kjeldahl method. From this analysis it is calculated that the material contains 0.15 mg of protein per ml, or 0.91 plus or minus 0.02 mg protein per mg of PPD–CT68.

(2) *Reproducibility from lot-to-lot.* The Master Batch of CT68 is stored as a dry powder in a desiccator at 5° C. A new lot apparently is prepared each year. Since the dried material has remained stable under the conditions of storage, each lot should be as uniform as it is possible to make a biological product. The protein nitrogen content of each lot is checked to assure conformation within the allowable limits described in the preceding section.

(3) *Standardization.* The Master Batch CT 68 has been compared exhaustively with PPD–S in humans with and without demonstrable disease. It was demonstrated that batch CT68 had about twice the antigenic potency of PPD–S. For this reason 2.5 working units (WU) of batch CT68 dispensed in phosphate buffered saline, pH 7.38, containing 5 ppm polysorbate 80 was estimated to be bioequivalent to 5 TU of PPD–S. There are 50,000 WU in 1 mg of PPD–CT68.

Each year a stock solution of batch CT68 (also called a "house standard") is prepared as a solution of 1 mg per ml and checked for proper nitrogen content. PPD–S is obtained from the Bureau of Biologics at similar intervals. The potency of the stock solution is compared to the Bureau of Biologics standard in 16 guinea pigs sensitized with heat-killed BCG. The stock solutions are considered to be acceptable if the results show its potency to be within 20 percent of the standard.

Bioequivalency to PPD–S is assumed for the 5 TU material on the basis of the guinea pig potency test. The 1 TU and 250 TU strengths are made by appropriate dilutions of the stock material. They are therefore calculated potencies and not actually bioequivalent to a standard. This would seem to be an acceptable compromise, however, since there are no reference standards for 1 TU and 250 TU PPD.

(4) *Stability.* The expiration date of the final product is stated to be 1 year from the date of manufacture. Data were submitted in the form of a publication by Landi in 1963 to indicate that the lyophylized material is stable for at least 24 months at temperatures of 5°, 24°, and 37°C. For this test, 2 ml of solutions of PPD in phosphate buffered saline with an inert base, polyvinylpyrrolidone, in concentrations of 1,000 TU and 50 TU per ml, were freeze dried in 10-ml vials. In the same paper it was shown that stock solutions were stable for 18 months at 5° and 24°C in concentrations of 10, 50, and 1,000 TU per ml dissolved in buffer containing 50 PPm of polysorbate 80. The test solutions were compared with dilutions of a reference standard at 50,000 TU per ml kept at 5°C, and the potency tests were performed in sensitized guinea pigs.

On the other hand, data were submitted which show that freshly prepared antigen in a diluent containing 5 ppm of polysorbate 80 produces a slightly larger reaction in hypersentitive guinea pigs than does an equivalent antigen 3 weeks old. Also, there are definite indications that 5 ppm of polysorbate-80 does not protect as well as 50 ppm in preventing the loss of potency owing to the adsorption of active protein to glass or plastic surfaces. Interpretation of the role of polysobate-80 is complicated by the observations of Wijsmuller and others of an in vivo effect of this material which makes for a qualitative as well as a quantitative change in the skin reaction.

(5) *Sensitization.* No data were submitted regarding induction or augmentation of immune response as a result of skin testing with batch CT68. It is reasonable to assume, however, that the discussion of this subject in the Generic Statement of PPD would apply equally well to this product.

(6) *Elimination by host.* Clearance from the skin test site within 24 hours has been demonstrated in guinea pigs. (Ref. 1). No data were available for humans.

(7) *Diluent.* The diluent is phosphate buffered saline, pH 7.38, containing 0.3 percent phenol as a preservative, and 0.0005 percent (5 ppm) polysorbate 80 as a stabilizing agent.

(ii) *Test method*—(1) *Application of test.* The Mantoux method is employed, which requires an intracutaneous injection of 0.1 ml of the material usually into the volar surface of the arm. It is a simple procedure, and it is accurate provided adequately calibrated syringes are used.

(2) *Measurement of test.* The positive reaction is round, indurated, and usually has a distinct border.

(3) *Quantitative dose response.* (see discussion under PPD Generic Statement). Data were submitted for CT68 to show that there is a quantatitive dose response which is linear over a small range of concentrations, in strengths of 1 to 5 TU.

(4) *Duration of reaction.* The test is read at 48 to 72 hours at which time the maximum reaction size usually has been attained.

(b) *Safety*—(1) *Host response.* No adverse reactions were reported to the manufacturer after many million test doses had been distributed. The only one of the listed reactions which is generally recognized is the rare severe skin reaction in a highly sensitive individual. The Panel found no information concerning potential carcinogenic, teratogenic or mutagenic effects from this product.

(ii) *Test material.* The bulk and final products are tested for sterility according to the Food and Drug Administration standards. These test methods indicate the absence of bacteria and fungi, and it is not likely that the material would contain other living agents. The absence of viable mycobacteria is assured by culture and guinea pig inoculation of the bulk concentrate. The standard safety tests according to FDA regulations are performed. There are no other tests for nonliving contaminants. A very large human experience indicates that the material is nontoxic.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* (a) The Panel recommends that this product should be placed in Category I, safe and effective. For future batches, the manufacturer should submit acceptable data based on scientifically sound methods to demonstrate effectiveness in humans (Ref. 2). These data should include for each batch:

(i) Comparison of the product with the standard by means of dose response curves.

(ii) Distribution of reaction sizes in persons known to be infected and in those presumed to be uninfected with *M. tuberculosis* and in addition in persons presumed to be infected with other mycobacteria.

(iii) Chemical characterization (including protein, carbohydrate, lipid and nucleic acid) should be performed on each batch to assess consistency of production.

(b) The labeling should indicate that the product is not stable in secondary containers (such as in a syringe). In the phrase "If a second test is employed, it should be repeated on the other forearm *preferably one month following the first test*" the last seven words should be deleted from the labeling. Consider replacing the term "atypical" with "other species of mycobacteria."

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(1) Landi, S., M. C. Tseng, and H. R. Held, "Retention of ^{14}C-Labeled Tuberculin in the Skin of Sensitized and Nonsensitized Animals," *Applied Microbiology*, 27:1085, 1974.

(2) Wijsmuller, G. and A. L. Bardine, "A Method of Characterizing Tuberculins," *American Review of Respiratory Diseases*, 105:736–746, 1972.

TUBERCULIN, PPD, MULTIPLE PUNCTURE DEVICE (HEAF) BY CONNAUGHT

1. *Product.* Tuberculin, PPD, Multiple Puncture Device (Heaf) manufactured by Connaught Laboratories, Ltd.

2. *Description.* The primary purpose of the Connaught multiple puncture Tuberculin, Purified Protein Derivative (PPD) device is to identify those individuals who have had an immunological experience with *Mycobacterium tuberculosis* or *Mycobacterium bovis* in a manner that can be applied by nonmedical personnel.

The Connaught product is a concentrated preparation of PPD containing 100,000 tuberculin units per ml. It is precipitated from tuberculin prepared in Long's synthetic medium in which the "Johnston" strain of *M. tuberculosis* is cultivated at 37° for 6 weeks in Povitsky bottles prior to steaming for 3 hours and filtration. The filtrate (tuberculin) is precipitated with 40 percent trichloracetic acid; this precipitate is dissolved and centrifuged to remove particulate matter and adjusted in volume to ¼ that of the original with M6 phosphate buffer. Phenol is added to a final concentration of 0.5 percent. This is the stock dilution of PPD. The final product is prepared from this solution and contains protein (estimated from the nitrogen and nucleic acid content) equivalent to 2 mg/ml Standard Tuberculin PPD.

The solution of PPD is applied with a sterile toothpick or platinum loop directly to the surface of the skin in a circular area approximately 10 mm in diameter. It is "injected" into the skin with a special instrument referred to as a "Sterneedle," with a controlled depth penetration by its six retractable points.

3. *Analysis*—(a) *Effectiveness.* Some data comparing the Heaf PPD to Mantoux tests suggest that the Heaf multiple puncture system can be as sensitive as the Mantoux test. In 155 tuberculosis patients, 95 percent were Heaf positive (Ref. 1). Other reports also indicate a high degree of co-positivity (Refs. 2 and 3). The degree of co-negativity in populations with a high prevalence of nontuberculous mycobacterial infections however, appears to be low (Ref. 4). Two studies suggest that the Heaf test is intermediate in strength to Mantoux tests using 5 TU and 250 TP (Refs. 3 and 5). The test sites were not randomized and the tests were not read blindly in any of the above studies.

(i) *Test material*—(1) *Chemical composition.* The protein, nucleic acid, glycerol, dibasic sodium phosphate and phenol content of the product are known. The exact chemical nature of the trichloracetic acid precipitated material is unknown.

(2) *Reproductibility.* No data were given.

(3) *Standardization.* Standardization is carried out against International Standard PPD–S in eight guinea pigs sensitized with BCG (fresh, freeze-dried or heat-killed) in 50 percent Freunds adjuvant. The number is in excess of that required by FDA regulations but is now known to be inadequate for the precision required (Ref. 6). The reaction sites are randomized but it is not known whether the reactions are read blindly. Evidence exists indicating that standardization in guinea pigs may not correlate with standardization in man (Ref. 7). However, the Connaught Heaf antigen is considered to be of acceptable potency if statistical analysis of the test guinea pig results show that the PPD content lies between 80,000 to 120,000 TU/ml, and when the micro-Kjeldahl test shows the concentration of protein to be equivalent to 2 mg/ml International Standard Tuberculin PPD.

(4) *Stability.* The material is relatively stable. Material stored at 5° C showed no detectable loss of potency after 24 months. There was slight loss of potency by 12 months in antigens stored at room temperature and at 37° C (Ref. 8).

(5) *Sensitization.* No data were provided.

(6) *Elimination by host.* No data were provided.

(7) *Diluent.* No diluent is used. The material itself is preserved in phenol which is not known to be sensitizing.

(ii) *Test method*—(1) *Application.* (a) No applicators are supplied with the Heaf material. It is applied to the skin by sterile toothpicks or flamed platinum loops, or by dipping the device in a small amount of the tuberculin. The "Sterneedle" is relatively simple to use although some persons find the manipulation of the unit, especially loading new disposable heads, more difficult than filling syringes, but the administration of the test is much simpler than the Mantoux test.

(b) As in other multiple puncture tests, the dosage of tuberculin cannot be measured. The risks vary from applying the tuberculin unevenly to applying none at all, especially with the cap-dip method recomended for large scale screening.

(2) *Measurement of the test reaction.* (a) The reaction is said to last 7 days. It is recommended that the reactions be read on the 4th and 7th days. The reactions generally progress from small papules at each of the six puncture points to coalesce and form a ring of solid circular induration. Of 7,628 individuals tested with the Sterneedle and Connaught PPD, 65.5 percent of the reactions appeared by the 3d day, 75.7 percent by the 5th, and 96.7 percent by the 7th (Ref. 11).

(b) The boundaries of the reaction are clear cut.

(3) *Quantitative dose response.* Quantitative dose response studies are not possible. Because of coalescence, the distribution of reaction sizes shows a break in continuity. Moreover, the dosage cannot be controlled.

(4) *Duration of reaction.* As noted above, the reaction lasts at least 7 days. Hsu believes that reactions that appear at 5 days or later are likely to be nonspecific (Ref. 9). Katz noted at least four nodules at 2 to 7 days (Ref. 4).

(b) *Safety*—(1) *Host response.* No adverse reactions have been reported from the many thousand vials that have been sold. The Panel found no information concerning potential carcinogenic, teratogenic or mutagenic effects from this product. However, one could anticipate that severe local reactions would be produced similarly to those resulting from the Sterneedle using Weybridge PPD. Of 2,284 positive reactions with this antigen administered by the Sterneedle, 264 (9.3 percent) were classified as severe. A total of 249 werecharacterized by vesiculation, blistering, ulceration or scabbing; 7 by intracutaneous hemorrhage; 4 by lymphangitis; and 4 by a severe reaction of unspecified nature. One case with constitutional syptoms was also noted (Ref.

10). There is also a study among tuberculosis children suggesting that necrosis is less frequent with the Sterneedle than with PPD applied by the Mantoux test (Ref. 4).

(ii) *Test material*—(1) *Sterility*—(*a*) *Bacterial.* The PPD concentrate is cultured for viable *M. tuberculosis* and is injected into guinea pigs. Cultures for other bacteria (aerobic and anaerobic) are done on the seed cultures, the filtrate, PPD concentrates, the final bulk concentrate, and contents of the final containers.

(*b*) *Other micro-organisms.* No tests are done for viruses, mycoplasma or fungi. It is highly unlikely that this material would contain other contaminants.

(2) *Purity.* Freedom from nonprotein contaminants is implied by its precipitation by trichloractetic acid. However, nucleic acid(s) would also be precipitated by this chemical and can be expected to be present in this product. No other data were provided.

(3) *Elimination by host.* No data provided.

(4) *Toxicity.* Following the distribution of many thousand vials containing 150 to 200 doses each over the past 5 years, no adverse reactions were reported except occasional severe "local" reactions. Animal tests in mice and guinea pigs performed with the final product as required by present FDA regulations also indicate that the product is not toxic.

(iii) *Test method.* The test produces very little pain or bleeding and is essentially noninjurious.

4. *Recommendations.* The Panel recommends that this product should be placed in Category IIIA, (to remain on the market pending more data), because adequate evidence of effectiveness of the present product in humans was not submitted. Since there is ample evidence in the literature (*see* the Generic Statement) that batches of tuberculin may vary widely in potency, it is necessary that each new batch be tested for potency (sensitivity) in infected humans. Potency tests in guinea pigs do not necessarily reflect potency in human beings. Since this test is to be used for screening purposes, then it is necessary to test only for sensitivity and not specificity. The Panel recommends that sensitivity be sufficient to elicit positive reactions in 100 percent of at least 50 persons who are known to have had bacteriologically confirmed tuberculosis and who are tuberculin positive as demonstrated by a simultaneous Mantoux test with 5 TU of PPD. One year is a reasonable time for the recommended testing to be accomplished.

References

(1) Coulter, W. W., and M. P. Martin, "Heaf Type Multiple Puncture Tuberculin Test Comparison With Mantoux and Vollmer," *Journal of the Louisiana State Medical Society*, 113:390–392, 1961.

(2) Andersen, S. R., and M. H. D. Smith, "The Heaf Multiple Puncture Tuberculin Test," *American Journal of Diseases of Children*, 99:764–769, 1960.

(3) Robins, A. B. and J. N. Daly, "Evaluation of the Heaf Tuberculin Test," *New England Journal of Medicine*, 262:1008–1012, 1961.

(4) Katz, J. "Comparative Studies of Tine, Heaf, and Mantoux Tests," *in Transcript of Proceedings of the Conference on Evaluation of Procedures for Tuberculin Testing*, sponsored by the Division of Biologics Standards and the American Thoracic Society, pp. 114–123, February 29, 1968.

(5) Smith, M. H. D. and S. Andersen, "Experience with Heaf Test in Children," *American Review Tuberculosis*, 78:319–320, 1958.

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(7) Hansen, O. G., K. Lindquist, and H. Waaler, "Assessment of the Potency of Tuberculin in Humans and Guinea-Pigs," *Bulletin of the World Health Organization*, 31: 171–182, 1964.

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(9) Hsu, K. H. K., "Further Studies on Heaf Testing," *American Review of Respiratory Diseases*, 84:129, 1961.

(10) Tuberculin Sub-committee, British Tuberculosis Association, "A Single Tuberculin Test for Epidemiological Use: A Comparison of the Mantoux and Heaf Tests," *Tubercle*, 40:317–335, 1959.

(11) Wright, W. P., "The Optimal Time of Reading the Heaf Test," *Canadian Journal of Public Health*, 53:150–157, 1962.

Recommendations for Regulations on Potency, Safety, Sterility, Purity, and Expiration Dating

The following Panel statements concern Part 610 of Title 21 of the Code of Federal Regulations as it pertains to the following products reviewed by the Panel:

Coccidioidin
Histoplasmin
Tuberculin, Old
Tuberculin, PPD
Diphtheria Toxin for Schick Test
Schick Test Control
Lymphogranuloma Venereum Antigen
Mumps Skin Test Antigen
Trichinella Antigen

Potency

Section 610.10 contains the general statement defining the requirements for potency and refers to § 600.3(s) of the same chapter for the definition of the term potency.

At the present time specific requirements are defined for Tuberculin and Diphtheria Toxin for Schick test. It is recommended that specific requirements be defined for each of the other products. Further the Panel recommends the development of a subsection on standardization of skin test products in animal models (Refs. 1 and 2). These procedures should be applied to each new batch.

General Safety

A proposed revision of § 610.11 is currently under review by the Food and Drug Administration. This expanded version details specific headings and clarifies various conditions for general safety that must be taken under consideration. This section is intended to deal directly with the question of general safety to ensure that the product does not contain extraneous toxic substances and for that reason defines the in vivo systems which must be employed.

The general safety test is a nonspecific test designed to rule out the possibility that some error has allowed a toxic material to be added to the product. Its only purpose is to provide additional assurance that no seriously harmful mistakes have been made in the preparation of a biological product up to and including the final preparation, dilution and bottling. It should be kept in mind that this is a general safety test, something to be applied to all final products in addition to tests for specific harmful effects, regardless of the nature of the product or the type of material used in its preparation. At the present state of the art, it is literally impossible to test the final product for all conceivable harmful effects.

Looked at in this light, the proposed general safety test seems adequate. A total of four animals representing two species is used in the first test. Truly heroic doses are given each animal. The dose of final product given a 22-gm mouse is equivalent to 1,136 ml for a 50 kg person; the dose given a 400 gm guinea pig is equivalent to 625 ml for a 50 kg person. In the toxicity tests for multiple puncture devices using dried tuberculin, the test doses are equivalent to giving a 50 kg person the material contained on 625 devices for a 400 gm guinea pig and 1,136 devices for a 22-gm mouse. Even slightly toxic additions to the product, given in such huge doses, can reasonably be expected to cause an animal to lose weight, sicken, or die.

Allowing repeat tests in case a failure occurs in the first, or first and second tests, is entirely reasonable. Death and illness among animals kept in large colonies not infrequently result from intercurrent disease, and the injection of completely harmless material in huge doses may be expected to affect some animals adversely. It should be noted that in case of failure to pass the first and second tests, the product must be given to a total of eight animals in the third test, all of which must remain healthy and not lose weight if the product is to be passed.

Sterility

Section 610.12 is the most detailed of the four sections to be covered by the panel. For its intended purpose, the section appears to be faulty in several respects. Three of the products under review are derived directly from animal sources and could harbor agents which under certain undefined conditions might have an undesirable effect on the individual receiving the product. Unless details are available elsewhere, the onus is on this panel to evaluate the necessity of having additional standards established for the production of each of the skin test antigens that are being scrutinized. In the case of histoplasmin,

coccidioidin, tuberculin-OT, tuberculin-PPD and diphtheria toxin, we are dealing with reagents which are cell free extracts of organisms. Section 610.12 adequately covers the requirements for sterility of each of these products. Lymphogranuloma venereum antigen, mumps virus antigen and Trichinella extract, on the other hand, are derived from animal sources. It is presumed that the end products have all been inactivated by various procedures but the regulations of the Bureau of Biologics do not stipulate specific tests which would ensure sterility including freedom from mycoplasma and viruses. For example, it is suggested that antigens requiring embryonated chicken's eggs for their production be checked to ensure that they are obtained from flocks that are free of avian leukosis virus, Rous inhibitory factor (Rif), and *Salmonella pullorum*.

It is recommended that specific requirements be defined for each product.

Purity

This topic is most difficult to define. If strict adherence is applied to the phrase "free from extraneous material," none of these skin test antigens meets the § 610.13 requirement. The regulations for each specific type of product need to define extraneous material as stringently as possible with the current state of the art.

In § 610.13(b) specific exceptions are allowed for bacterial, viral and rickettsial antigens and for toxins in the test for pyrogenic substances. The Panel recommends that fungal antigens also be included in this category.

In summary, this section of the regulations requires clarification, and the recommendation is made that it be revised.

Standard Preparations

The availability of § 610.20 Reference Standards at the national level is basic to the continued production of effective quality preparations for general use. It is reasonable to suspect that all existing Reference Standards are subject to some biological deterioration, yet, at present there are no systematic attempts to test the reference standards to determine their duration of potency. This Panel recommends the initiation of studies to determine methods by which reference standards can be maintained reliably for long periods of time.

Dating Period Limitations

Section 610.50 refers to products for which an official standard of potency is prescribed in § 610.20 or § 610.21. Such products include only diphtheria toxin for the Schick test, Old Tuberculin and Tuberculin, PPD. For products not subject to official potency tests, dating period limitations begin from the date of extraction, the date of solution, or the date of cessation of growth, whichever is applicable. Sections 610.51 and 610.52 indicate that, except as otherwise provided, products may be held in cold storage by the manufacturer at a temperature not above 5° C for 1 year and at a temperature not above 0° C for 2 years. The dating period begins on the date of manufacture, except when the material is stored according to the above temperature, at which point it may be dated from the date of issue from the manufacturer's cold storage. If it is held in a manufacturer's cold storage beyond the period indicated above, then the dating period has to be reduced for the period in excess of that allowed by manufacturer's cold storage. In the latter circumstances the storage temperatures and times allowed the manufacturer vary from the general provisions of § 610.51. In such circumstances, dating after manufacture or withdrawal from the manufacturer's storage is given first, and in parenthesis the manufacturer's allowable storage temperatures and times are given. For example, "3 years (5° C, 1 year)" means that the product will have an expiration date 3 years after the manufacturer takes it out of storage, and that the storage period is limited to 5° C for 1 year. The Panel believes that the dating periods in § 610.53 are reasonable but recommends that they should be established for each manufacturer's product by scientific investigation.

Additional Recommendations

In addition to the specific recommended changes, a new regulation should be developed concerning "Standardization of Skin Test products in Animals and Man" (Refs. 1 and 2). Also, tests for specific hazards need to be developed for each product on an individual basis and applied at the most advantageous point in the manufacturing process, which will usually be prior to the final preparation.

References

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Thimerosal as a Preservative

A preservative is a substance added to a product for the purpose of destroying or inhibiting the multiplication of microorganisms. As described in the introduction, preservatives used in the the manufacture of diagnostic skin test antigens should neither sensitize the immune system nor elicit any other local or systemic host reaction.

Hypersensitivity to thimerosal and other mercurial compounds has been recognized for many years (Refs. 1, 2, and 14). It is receiving renewed attention because of the inclusion of thimerosal as a preservative in many licensed biologic products (Refs. 3 through 13 and 16). Of the approximately 180 licensed products containing thimerosal, 15 contain thimerosal at a concentration of 1:20,000 (w/v), 3 at 1:30,000, and 1 at 1:40,000. All others are preserved with 1:10,000 except for a few dried products with higher concentrations.

Products containing thimerosal include allergenic extracts, antisera, antivenins, bacterial, bedsonial and viral vaccines, immune serum globulins, antitoxins and toxoids; skin tests for bacteria, fungi and viruses; and miscellaneous items such as laboratory diagnostic agents. Most are beneficial or instructive only if injected intracutaneously, subcutaneously, intramuscularly or intravenously.

Since many persons, particularly children, are routinely injected with a number of these products, there is the possibility that any sensitizing property of thimerosal in any one product could be cumulative following the administration of several products. Thus, the danger of sensitivity to mercury may be considerably greater than in years prior to the frequent use of many products preserved with derivatives of this element. Additional opportunities for sensitization may also be provided by ingestion of foods contaminated by certain industrial mercurial wastes, e.g., fish—or merely by topical application. The few published reports could be portents of more serious difficulties yet to come in a population frequently injected with products preserved with thimerosal.

Ellis and Robinson (Ref. 2) first noted cutaneous sensitivity to thimerosal and other mercurial compounds in 1942. Five years later Ellis (Ref. 1) defined a sensitizing factor in thimerosal as the thiosalicylate. Little further work was done until 1963 when Epstein (Ref. 3) demonstrated that hypersensitivity to mercury compounds might be manifested as delayed hypersensitivity reactions. In 1969 Reisman (Ref. 11) working with patients in an allergy clinic at Brooke General Hospital found 10 patients within 6 months whose reactions were due to the thimerosal (1:10,000) preservative in the allergenic testing solutions. He noted the number of vaccines—both licensed and experimental—received by service personnel and recommended that "in view of the availability of phenol which rarely, if ever, sensitizes, there seems to be little reason to continue to use Merthiolate as a preservative in allergy extracts."

After an investigation of the cause of side reactions to tetanus toxoid among military personnel in Sweden, Hansson and Möller (Ref. 6) further investigated thimerosal, the toxoid preservative, as a possible cause of these reactions. They patch tested three groups with 0.1 percent thimerosal. Healthy recruits were in Group A (203) and B (29). Reactions occurred among 33 (16 percent) and 10 (35 percent), respectively. Of 247 patients in Group C with suspect contact dermatitis, 89 (36 percent) reacted to standard allergens and 10 (7 percent) to the thimerosal. In 51 patients with various dermatoses (Group D) none reacted to thimerosal at 1.0 percent. Patients in groups C and D were two to three decades older than the healthy recruits in groups A and B. From these findings the authors deduced that age might be a factor in thimerosal sensitivity. It is also notable that subjects in Group B were trained as

hospital orderlies and in addition to receiving service immunizations were quite likely exposed to mercurial derivatives in other ways. In a later study conducted by Hansson and Möller (Ref. 7), 15 percent of a group of 412 males reacted positively to thimerosal, 0.1 percent in saline at 48 and 72 hours.

This study doubtless led to a similar study, "Coccidioidin and Merthiolate in Previously Sensitized Animals (Ref. 4)." A statistical analysis of skin test responses was used to detect fine differences when thimerosal was (1) incorporated into a skin test material or (2) used as a preservative in a sensitizing material. Skin tests were also done with the basic medium in which the coccidioidins were prepared, with and without the thimerosal preservative. The authors concluded from this study that "the use of Merthiolate in human skin test materials should be given careful consideration."

On the other hand skin test antigens containing thimerosal have been given to large numbers of persons without eliciting excessive positive skin reactions attributable to thimerosal. For example, in many studies, histoplasmin preserved with thimerosal has produced virtually no reactions (Refs. 17 and 18).

As demonstrated in guinea pigs, reactions to skin test antigens containing thimerosal are significantly enhanced by prior exposure (of the individual) to thimerosal. If a positive reaction is produced in response to an antigen preserved in thimerosal it cannot be known whether the reaction was produced by the antigen or the thimerosal. The presence of thimerosal did not cause a decrease in the size of reactions to coccidioidin in a study in animals but reactions to coccidioidins containing thimerosal were generally greater than to those without thimerosal (Ref. 4). This finding may be most important in the indeterminate region of a skin test reaction where a truly negative reactor may be classified as positive because of the added reaction caused by thimerosal.

Recommendations

There is now evidence that thimerosal may (1) induce cell mediated hypersensitivity and (2) affect the size of the delayed skin test reaction in some subjects. Therefore, (1) when other preservatives have been demonstrated to be safe, effective, nonsensitizing (as well as not causing deterioration of the product, products containing thimerosal should be discontinued; and (2) a search for safe, effective and nonsensitizing preservatives as alternatives for thimerosal should be initiated.

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Statement Concerning Skin Test Reagents Employed for Assessing Immunocompetence

A variety of skin test antigens is frequently employed in tests to assess immunocompetence. For this reason the Panel on Review of Skin Test Antigens submits the following statement.

The rationale for using skin tests for this purpose is based on the presumption that the individual being tested has had previous experience with the antigenic material. A negative Schick Test implies that the individual is capable of producing antibodies. A positive delayed-type skin reaction implies that the cellular immune system is competent; a negative test implies that the individual is immunologically naive or deficient. Currently there is no single antigen which can be used to evaluate all aspects of immune function. Therefore, a battery of skin test antigens is employed for assessing cellular immunocompetence. The following materials have been included in this battery:

(1) Tuberculin, PPD.
(2) Candida extract (oidiomycin).
(3) Trichophytin.
(4) Streptococcus antigen (Streptokinase/streptodornase).
(5) Mumps skin test antigen.

In geographical areas where there is a high incidence of reactivity to certain antigens such as coccidioidin and histoplasmin, these antigens may serve as additional reagents.

It is the Panel's charge to review these products for safety and effectiveness for their labeled uses. Since the use in measuring delayed hypersensitive immunocompetency is not part of the present licenses, the Panel can only recognize that such use exists based upon reports in the literature, letters from physicians, and personal experience. A new license application or license amendment from the producers should be submitted for this new indication with supportive data on effectiveness and standardization. The Panel recommends that this be accomplished.

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The Commissioner is responding to the Panel's recommendations as follows:

1. The Panel recommended that skin test antigens be grouped into regulatory categories, as follows:

(a) *Category I. Biological products determined to be safe and effective and not misbranded and should continue in interstate commerce.* Tuberculin, Old (multiple puncture device), Lederle Laboratories, license No. 17; Tuberculin, Old (multiple puncture device), Institut Merieux, license No. 384; Tuberculin, PPD (Mantoux intradermal), Connaught Laboratories, Ltd., license No. 73; Tuberculin, PPD (Mantoux intradermal), Parke, Davis and Co., license No. 1; Tuberculin PPD (multiple puncture device), Parke, Davis and Co., license No. 1.

The Commissioner agrees with the Panel's findings and recommendations concerning these drugs and, in accordance with § 601.25(f)(1), hereby proposes that they should remain in interstate commerce and that their licenses should remain in effect. Comments and/or additional data on the Category I status of these products are invited.

(b) *Category II. Biological products determined to be unsafe or ineffective or to be misbranded and should not continue in interstate commerce.* Lymphogranuloma Venereum Antigen, Lederle Laboratories, license No. 17; Trichinella Extract, Lederle Laboratories, license No. 17.

The Commissioner agrees with the Panel's findings and recommendations concerning these drugs. However, proceedings toward revocation to implement the Commissioner's conclusion are unnecessary since, at the request of Lederle Laboratories, the licenses for Lymphogranuloma Venereum Antigen and Trichinella Extract were revoked on August 11, 1975 and March 16, 1976 respectively.

(c) *Category IIIA.—Biological products for which available data are insufficient to classify their safety and effectiveness but which may remain in interstate commerce pending completion of testing.* Coccidioidin, Cutter Laboratories, Inc., license No. 8; Coccidioidin, Iatric Corporation, license No. 416; Histoplasmin, Parke, Davis and Company, license No. 1; Histoplasmin, Bureau of Laboratories, Michigan, license No. 99; Diphtheria Toxin for Schick Test, Massachusetts Public Health Biologics Laboratories, license No. 64; and Tuberculin, PPD (multiple puncture device), Connaught Laboratories, Ltd., license No. 73.

The Commissioner agrees with the Panel's findings and recommendations concerning these drugs and hereby proposes, in accordance with § 601.25(f)(3), that products listed in Category IIIA remain on the market and their licenses remain in effect on an interim basis provided that acceptable data based on scientifically sound studies (as recommended in the Panel report) be submitted to demonstrate efficacy in humans. Such studies shall be in accordance with the Panel's recommendations and § 601.25(h), and shall be completed within 2 years of publication of the final order. Failure to submit adequate data, in the form of a license amendment, within the specified time will result in the publication of a notice of an opportunity for hearing to revoke the product license. Comments and/or additional data on the Category IIIA status of these products are invited.

Since the Panel's conclusions concerning the effectiveness of Category IIIA drugs is a material fact within the meaning of 21 U.S.C. 321(n) and 21 CFR 1.21 the failure to disclose which causes these drugs to be misbranded, the Commissioner also proposes that the circular and the promotional material for these drugs must have a prominent boxed statement referencing the need for further data to fully establish effectiveness. Moreover, the Commissioner also proposes that a written informed consent be obtained from participants in the requisite additional studies, that an explanation of the product and the purpose of the study is given and that a clear opportunity to refuse to participate in the study is presented. Accordingly, § 601.25 is proposed to be amended to require this information.

(d) *Category IIIB.—Biological products for which available data are insufficient to classify their safety and effectiveness and should not continue in interstate commerce.* Product licenses are to be revoked for the following products pending completion of testing and label revision, as recommended in the Panel report: Histoplasmin (multiple puncture device), Lederle Laboratories, license No. 17; Mumps Skin Test Antigen, Eli Lilly and Company, license No. 56; Diphtheria Toxin for Schick Test, and Diphtheria Toxoid for Schick Test Control, Texas Department of Health Resources, license No. 121; Diphtheria Toxin for Schick Test, Parke, Davis and Company, license No. 1; Lymphogranuloma Venereum Antigen, E. R. Squibb and Sons, Inc., license No. 52, Tuberculin, Old, Parke, Davis and Company, license No. 1; Tuberculin, Old, Eli Lilly and Company, license No. 56; Tuberculin, Old, Massachusetts Public Health Biologics Laboratories, license No. 64; Tuberculin, Old, Texas Department of Health Resources, license No. 121. The Panel recommended a period of 2 years for completion of testing.

The Commissioner agrees with the Panel's findings and recommendations concerning these drugs and, in accordance with §§ 601.5 and 601.25(f)(3), intends to publish a notice of opportunity for hearing to revoke the licenses for these products by or before October 31, 1977. Interested persons may comment and/or submit additional data in response to the notice. In addition, with respect to Diphtheria Toxin for Schick Test and Tuberculin, Old, processed by Parke, Davis & Co., and Lymphogranuloma Venereum Antigen, processed by E. R. Squibb & Sons, Inc., the Commissioner also intends to base revocation upon the fact that manufacture has discontinued to such an extent that a meaningful inspection cannot be made, as prescribed in § 601.5(b)(2). These revocation procedures will be unnecessary for Tuberculin, Old, manufactured by the Texas Department of Health Resources under license No. 121 and Histoplasmin manufactured by Lederle Laboratories under license No. 17 because, at the request of the licensees, the licenses were revoked on July 14, 1977 and August 11, 1975, respectively.

2. The Panel noted that specific requirements, called "additional standards," for Tuberculin (21 CFR 650.10–650.15) and Diphtheria Toxin For Schick Test (21 CFR 650.1–650.7) are published in the Code of Federal Regulations. They recommended that similar additional standards be published for Coccidioidin, Histoplasmin, Schick Test control, Lymphogranuloma Venereum Antigen, Mumps Skin Test Antigen and Trichinella Extract.

The Commissioner notes that there are existing standards, called "minimum requirements," for three of these products and the licenses for the others have been or will be proposed to be revoked. Accordingly, it appears that published "additional standards" are not of high priority at this time but will be codified when feasible and if necessary.

3. The Panel recommended that § 610.1 be amended to prescribe specific tests to insure sterility, including freedom from mycoplasma and viruses, which may be present in licensed Lymphogranuloma Venereum Antigen, Mumps Skin Test Antigen, and Trichinella Extract products.

The Commissioner again notes that these licenses have been or will be proposed to be revoked. The Panel recommendation will be reconsidered if any new license applications for similar products are received and approved.

4. The Panel recommended that § 610.13 be amended to define "extraneous material" for each product and that § 610.13 be amended to include fungal antigens in the list of products exempted from the requirements of a pyrogen test.

The Commissioner recognizes that some extraneous material is unavoidable in the manufacturing process. The term "extraneous material" is not intended to include such contaminants. Accordingly, to clarify the regulation, the Commissioner proposes to amend § 610.13 to specify that products need to be free from avoidable, extraneous material.

The Commissioner also recognizes that fungal antigens are expected to contain pyrogens. However, the listing of "diagnostic substances" in § 610.13(b) as being among those products exempted from the requirement of a pyrogen test includes fungal antigens. Accordingly, there is no need to amend § 610.13(b).

5. The Panel recommended (a) initiation of studies to determine methods by which reference standards can be maintained reliably for long periods of time, (b) amendment of the biologic regulations to include a new regulation concerning standardization of skin test products in animals and man, and (c) amendment of § 610.53 concerning dating period for specific products to establish a dating period determined by scientific investigation for each manufacturer's product.

The Commissioner accepts the Panel's recommendations concerning the initiation of studies to assure that reference standards can be maintained reliably for long periods of time, and the issuance of a regulation on the standardization of skin test products. Some studies responsive to the Panel's recommendations are in progress, and others will begin as soon as feasible and as priorities permit. When sufficient data from these studies will permit, a proposal on standardization will be published. The Commissioner advises that § 610.53 is intended to prescribe dating periods for each product type rather than for each manufacturer's product. The regulation identifies the dating period permitted and includes a margin of safety. Data have been pre-

viously submitted by each manufacturer demonstrating stability within these limits. However, the Commissioner will consider data supporting a license amendment for a separate or different dating period for the licensed product manufactured by any particular licensee.

6. The Panel noted that there is evidence that thimerosal (a mercurial preservative) may induce cell mediated hypersensitivity to itself and affect the size of the delayed skin reaction in some subjects. This results, in effect, in a false positive reaction. Therefore, when other preservatives have been demonstrated to be safe, effective, and nonsensitizing, products containing thimerosal should be discontinued. Accordingly, a search for safe, effective, and nonsensitizing preservatives as alternatives for thimerosal should be initiated.

The Commissioner agrees with this recommendation. There are only a few preservatives that have been found to be safe and effective for use in injectable biological products. Other substances, frequently employed as preservatives in nonbiological products, are unacceptable because of interactions with the proteinaceous components of biologicals and because they may also be sensitizing. Due to these problems, extensive research is required to demonstrate the propriety of the new preservative in each biological product. Therefore, when other preservatives are shown to be safe and effective in ongoing studies of biological products, regulations proposing their use in place of thimerosal will be considered.

7. The Panel noted that the following antigens, among others, are frequently employed in tests to assess immunocompetence and recommended that they be licensed for this purpose, subsequent to the submission of effectiveness and standardization data:

(a) Tuberculin, PPD.
(b) Candida extract (oidiomycin).
(c) Trichophytin.
(d) Streptococcus antigen (Streptokinase/Streptodornase).
(e) Mumps Skin Test Antigen.

The Commissioner recognizes that some physicians, during their normal practice of medicine, use these products to assess immunocompetence. However, these products are not now labeled for this purpose by the manufacturer, and no data relating to the effectiveness of the products for this use have been submitted to FDA to support licensure although there is a clear need for products to assess immunocompetence. The Commissioner encourages the acquisition of data that would evaluate this use. However, until such data are provided, the labeling for these products must not represent or suggest usefulness for immunocompetence testing. Continued use of statements for this use will result in regulatory action.

8. The Panel suggested that the Biologics regulations governing the manufacture of Tuberculin be amended to acquire that:

For each bulk lot of tuberculin material for use on multiple puncture devices for screening purposes, the product sensitivity shall be demonstrated to be sufficient to elicit positive reactions in 100 percent of at least 50 persons who are known to have had bacteriologically confirmed tuberculosis and who are tuberculin positive as demonstrated by a simultaneous Mantoux test with 5 TU of Tuberculin, Purified Protein Derivative * * *

For each bulk lot of tuberculin material for use by the Mantoux method, effectiveness data in humans should be supplied to include (1) comparison of the product with the standard by means of dose response curves, and (2) distribution of reaction sizes in persons known to be infected and in those presumed to be uninfected with Mycobacterium tuberculosis and in addition in persons presumed to be infected with other mycobacteria. Chemical characterization (including protein, carbohydrate, lipid and nucleic acid content) should be performed on each batch to assess consistency of production * * *.

The Commissioner accepts the Panel's recommendations and proposes to incorporate these suggestions by amending Part 650.

9. The Panel recommended that evidence be furnished for the long-term safety of the red dye, FD&C Red No. 2, or that the dye be eliminated from the product Histoplasmin, produced by Parke, Davis and Company. This color was originally included in the product in a concentration of 1 to 7,000 or 0.143 micrograms per intracutaneous dose, to ensure proper quality control in the filling operation by making visible the extremely small volume of fill.

The Commissioner advises that action has been taken to reformulate and eliminate the color from the product. Various alternatives are being explored to ensure proper quality control during filling.

The Commissioner has carefully considered the environmental effects of the proposed regulation and, because the proposed action will not significantly affect the quality of the human environment, has concluded that an environmental impact statement is not required. A copy of the environmental impact assessment is on file with the Hearing Clerk, Food and Drug Administration.

Therefore, under the Federal Food, Drug, and Cosmetic Act (secs. 201, 502, 505, 701, 52 Stat. 1040–1042 as amended, 1050–1053 as amended, 1055–1056 as amended by 70 Stat. 919 and 72 Stat. 948 (21 U.S.C. 321, 352, 355, 371)), the Public Health Service Act (sec. 351, 58 Stat. 702 as amended (42 U.S.C. 262)), and the Administrative Procedure Act (secs. 4, 10, 60 Stat. 238 and 243 as amended (5 U.S.C. 553, 702, 703, 704)); and under authority delegated to him (21 CFR 5.1), the Commissioner is proposing that Parts 601, 610 and 650 in Subchapter F of Chapter I of Title 21 of the Code of Federal Regulations be amended as follows:

PART 601—LICENSING

1. In Part 601 by amending § 601.25(h) by revising the paragraph heading and adding new (h)(4) and (5) to read as follows:

§ 601.25 Review procedures to determine that licensed biological products are safe, effective, and not misbranded under prescribed, recommended or suggested conditions of use.

* * * * *

(h) *Additional studies and labeling.* * * *

(4) Labeling and promotional material for products requiring additional studies shall bear a box statement in the following format:

> Based on a review by the Panel on Review of (insert name of appropriate panel) and other information, the Food and Drug Administration has directed that further investigation be conducted before this product is determined to be fully effective for the labeled indication(s).

(5) A written informed consent shall be obtained from participants in the requisite additional studies explaining the nature of the product and the investigation. The explanation shall consist of such disclosure and be made so that intelligent and informed consent be given and that a clear opportunity to refuse is presented.

PART 610—GENERAL BIOLOGICAL PRODUCTS STANDARDS

2. In Part 610 by amending § 610.13 by revising the introductory paragraph to read as follows:

§ 610.13 Purity.

Products shall be free from extraneous material except that which is unavoidable in the process in the approved license. In addition, products shall be tested as provided in paragraphs (a) and (b) of this section.

* * * * *

PART 650—ADDITIONAL STANDARDS FOR DIAGNOSTIC SUBSTANCES FOR DERMAL TESTS

3. In Part 650: a. By amending § 650.13 by adding new paragraph (c) to read as follows:

§ 650.13 Production.

* * * * *

(c) *Chemical characterization.* Each batch of powdered tuberculin material shall be chemically characterized, including protein, carbohydrate, lipid and nucleic acid content to assess consistency of production.

b. By amending § 650.14, by revising the title to read as follows:

§ 650.14 Potency testing in animals.

* * * * *

c. By redesignating existing § 650.15 as § 650.16 *Equivalent methods.*

d. By adding new § 650.15 to read as follows:

§ 650.15 Potency testing in humans.

(a) The sensitivity of each batch of tuberculin material for use on multiple puncture devices for screening purposes shall be demonstrated to be sufficient to elicit positive reactions in 100 percent of at least 50 persons who are known to have had bacteriologically confirmed tuberculosis and who are tuberculin positive as demonstrated by a simultaneous Mantoux test with 5 TU of Tuberculin, Purified Derivative.

(b) The product effectiveness of each batch of tuberculin material for use by the Mantoux method shall include comparison of the product with the standard by means of (1) dose response curves, and (2) distribution of reaction sizes in persons presumed to be uninfected with *Mycobacterium tuberculosis* or other mycobacteria, in persons known to be infected with *M. tuberculosis*, and in persons presumed to be infected with other mycobacteria.

Interested persons may, on or before November 29, 1977, submit to the Hearing Clerk (HFC–20), Food and Drug Administration, Rm. 4–65, 5600 Fishers Lane, Rockville, MD 20857, written comments regarding this proposal. Four copies of all comments shall be submitted, except that individuals may submit single copies of comments, and shall be identified with the Hearing Clerk docket number found in brackets in the heading of this document. Received comments may be seen in the above office between the hours of 9 a.m. and 4 p.m., Monday through Friday.

* * * * *

NOTE.—The Food and Drug Administration has determined that this document does not contain a major proposal requiring preparation of an inflation impact statement under Executive Order 11821 and OMB Circular A–107. A copy of this inflation impact assessment is on file with the Hearing Clerk, Food and Drug Administration.

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Dated: September 19, 1977.

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DONALD KENNEDY,
Commissioner of Food and Drugs.

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